Rab proteins, their effectors and GAPs in neuroendocrine exocytosis

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy by

Mark Thomas Wild Handley

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Contents

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Abstract

Rab proteins are small GTPases of the Ras superfamily implicated in the control of membrane trafficking. Secretory granule exocytosis is a highly regulated process by which various molecules and macromolecules may be expelled from secretory cells. The Rab proteins most directly linked to roles in the trafficking and tethering/docking of secretory granules, and in their fusion with the plasma membrane, are isoforms of Rab3 and Rab27. In this study, the recruitment of Rab3A and Rab27A and several of their effectors to granules, the dynamics of their granule association, their function, and their regulation, were explored in PC12 cells.

Endogenous Rab3A colocalised with the secretory granule marker secretogranin II (SGII) and expressed EGFP-Rab3A and ECFP-Rab27A colocalised with one another. The extent of colocalisation between EGFP-Rab3A or EGFP-Rab27 and SGII increased after longer times post transfection suggesting that these Rab proteins are preferentially recruited to newly synthesised granules. Following the release of immature secretory granules from the trans-Golgi network, Rab3A and Rab27A became associated with the immature granules after a lag period of around 20 minutes. Fluorescence recovery after photobleaching (FRAP) experiments indicated that Rab3A but not Rab27A appears to be continually exchanged between granules and cytosol, and that this exchange occurs in an HSP90-independent manner. The Rab proteins were not found to disperse from exocytotic sites on cellular stimulation.

EGFP-tagged forms of the Rab effectors Rabphilin, Granuphilin and Noc2 were found to colocalise with secretory granules labelled with either mRFP-Rab3A or mRFP-Rab27A. Rabphilin but not Granuphilin or Noc2 was rapidly exchanged between granules and cytosol. Granuphilin, but not Rabphilin or Noc2, was predominantly localised to granules at the periphery of cells. Noc2, but not Rabphilin or Granuphilin, was found to inhibit secretion on moderate overexpression, and this inhibition was attenuated when its binding to Rab proteins was disrupted. Overexpression of a 'gain of function' mutant of the novel Rab interactor Munc18-1 was found to increase the density of EGFP-Rab3A and EGFP-Rab27A labelled secretory granules at the periphery of cells, while it did not appear to affect cortical actin. This effect was not seen where a 'double mutant' with reduced affinity for the 'closed' form of Syntaxin was expressed.

The properties of a series of putative GTPase activating proteins (GAPs) for Rab3A and Rab27A were analysed by screening for their capacity to disperse EGFP-tagged Rabs from secretory granule membranes. A GAP previously reported to act on Rab3A, p130 Rab3GAP, was found to lack this capacity, while GAPs reported to act on Rab27A, TBC1D10A and TBC1D10B, were found to act non-specifically. In contrast, a candidate Rab3GAP, RUTBC1, was found to specifically disperse EGFP-Rab3A, to colocalise with Rab27A on secretory granules, and to suppress the inhibitory effects of EGFP-Rab3A overexpression on secretion.

Publications

Handley, M.T., Haynes, L.P., and Burgoyne, R.D. (2007). Differential dynamics of Rab3A and Rab27A on secretory granules. Journal of Cell Science *120*, 973-984.

Graham, M.E., ***Handley, M.T.**, Barclay, J.W., Ciufo, L.F., Barrow, S.L., Morgan, A., and Burgoyne, R.D. (2008). A gain-of-function mutant of Munc18-1 stimulates secretory granule recruitment and exocytosis and reveals a direct interaction of Munc18-1 with Rab3. The Biochemical Journal *409*, 407-416.

**joint first author*

Abbreviations

α-SNAP	α-Soluble NSF attachment protein
AMPA	alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
Arf	ADP-rybosylation factor
Arl	Arf-like protein
ATP	Adenosine triphosphate
CaMKII	Calcium-Calmodulin-activated kinase
cAMP	cyclic Adenosine monophosphate
Cdc42	Cell division cycle 42 protein
cDNA	complementary DNA
CMV	Cytomegalovirus
COPI	Coatamer protein complex I
COPII	Coatamer protein complex II
CSP	Cysteine string protein
Cys	Cysteine
DMPP	1,1-dimethyl-4-phenylpiperazinium iodide
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
ECFP	Enhanced cyan fluorescent protein
EDTA	Ethylene diamine tetra-acetic acid
EGFP	Enhanced green fluorescent protein
ER	Endoplasmic reticulum
ERES	ER exit sites
ERGIC	ER-Golgi intermediate compartments
ERM	Ezrin/radixin/moesin
EYFP	Enhanced yellow fluorescent protein
F-Actin	Filamentous actin
FM	Fei Mao
FRAP	Fluorescence recovery after photobleaching
GA	Geldanamycin
GAP	GTPase-activating protein
GCR	Glucocorticoid receptor
GDF	GDI-displacement factors
GDI	GDP dissociation-inhibitor
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GGTase	Geranylgeranyl transferase
GluR1	Glutamate receptor subunit 1 protein
GLUT4	Glucose transporter 4
GppNHp	Guanyl-5'-y1-beta-gamma-imidodiphosphate
GST	Glutathione-s-transferase
GTP	Guanosine triphosphate
HEPES	Hydroxyethyl-piperazine-N'-2-ethanesulfonic acid
hGH	human Growth hormone
HSP90	Heat-shock protein-90
LCEA	Last common eukaryotic ancestor
LTP	Long-term potentiation
M	Molar
MDCK	Martin Darby Canine kidney
mg	Milligrams
µg	Micrograms
µl	Microlitres
µM	Micromolar
ml	Millilitres
mM	Millimolar
MPR	Mannose-6-phosphate receptor
mRFP	monomeric Red fluorescent protein

Munc13	Mammalian uncoordinated 13 protein
Munc18-1	Mammalian uncoordinated 18 protein
MyRIP	Myosin VIIA and Rab interacting protein
nanoM	Nanomolar
NCS	Neuronal calcium sensor
NGF	Nerve growth factor
nm	Nanometers
NPY	Neuropeptide Y
NRK 52E	Normal rat kidney 52E cells
NSF	N-ethylmaleimide-sensitive fusion protein
PBS	Phosphate-buffered saline
PBT	PBS with BSA and Triton-X-100
PC12	Pheochromocytoma 12
PCR	Polymerase chain reaction
PI	Phosphatidylinositol
PI(3)P	Phosphatidyl inositol 3-phosphate
PI3K	Phosphatidyl inositol 3-OH kinase
PIP2	Phosphatidylinositol (4,5) bisphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
ppANF	prepro-Atrial natriuretic factor
PPF	Paired-pulse facilitation
PS	Phosphatidylserine
Rab	Rat brain protein
Rac	Ras-related C3 botulinum toxin substrate protein
Ran	Ras-like nuclear protein
Ras	Raus sarcoma protein
RBD	Rab-binding domain
REP	Rab escort protein
Rho	Ras homolog
Rheb	Ras homolog enriched in brain
RIM	Rab-interacting molecule
Rin	Ras-like protein expressed in neurons
Rit	Ras-like protein expressed in many tissues
RNA	Ribonucleic acid
ROI	Region of interest
Sar1	secretion-associated and Ras-related protein 1
s.e.m.	standard error from the mean
SDM	Site-directed mutagenesis
SDS	Sodium dodecyl sulphate
Sec	Secretory (protein)
SGII	Secretogranin II
siRNA	small interfering RNA
Slac2	Synaptotagmin-like lacking C2 domains
Slp	Synaptotagmin-like protein
SM	Sec1/Munc18-like
SNAP-25	Synaptosomal associated protein of 25kDa
SNARE	Soluble N-ethyl maleimide-sensitive attachment protein receptor
STP	Short-term plasticity
TAE	Tris-acetate EDTA
TBC	Tre-2, Bub-2, Cdc16
TGN	Trans-Golgi network
TIRFM	Total internal reflection microscopy
TRAPP	Transport protein particle
Triton-X-100	Octylphenoxy polyethoxyethanol
v/w	volume by weight
VAMP	Vesicle-associated membrane protein
Ypt	Yeast protein transport protein

Chapter 1:

Introduction

1.1 Origins of exocytotic pathways

Membranes composed of phospholipid bilayers serve to compartmentalise cellular function in eukaryotes (Lowe and Barr, 2007). It is hypothesised that the eukaryotic cytomembrane system developed from a process of reversible plasma membrane invagination and vesiculation used by early relatives of protoeukaryotes for endocytotic import and intracellular digestion of extracellular materials and the subsequent exocytotic expulsion of the resulting residues (De Duve and Wattiaux, 1966; de Duve, 2007). Further, it is thought that the selective advantages conferred by this process on heterotrophic cells, reliant on these materials for nutrition, were significant. Not only might the internalisation of nutrients have led to improved efficiency in their utilisation, endocytosed material might also have provided a source of nourishment for cells where this became scarce externally (Cavalier-Smith, 2002; de Duve, 2007).

The refinement of early endomembrane systems of nutrient uptake and processing, and the emergence of specialised membrane compartments serving wider cellular roles, are suggested to have been intrinsic to eukaryote evolution (Dacks and Field, 2007; de Duve, 2007). Had they been peripheral to the process, eukaryotic lineages lacking basic membrane trafficking componentry might exist. However, the available phylogenetic evidence indicates that in addition to the digestive and recycling machinery of endosomes and lysosomes, the last common eukaryotic ancestor (LCEA) also possessed the biosynthetic and secretory

machinery of endoplasmic reticulum (ER) and Golgi (Dacks and Field, 2007). Several theories of eukaryogenesis implicate complex membrane trafficking, and in particular the process of phagocytosis, in the adoption by protoeukaryotes of the endosymbiont progenitors of mitochondria and plastids, critical events thought to have occurred after levels of atmospheric oxygen began to rise on Earth ~2.4 billion years ago (Cavalier-Smith, 2002; Bekker *et al.*, 2004; de Duve, 2007).

A recently proposed hypothesis suggests that an ancestral secretory pathway predates the emergence of the phagosomal pathway in protoeukaryote evolution, and may therefore explain its presence in LCEA (Jekely, 2003). This hypothesis is based on the phylogeny of small GTPases, and analysis suggesting that Sar1/Arf proteins diverged from other GTPases prior to the divergence of Ran, and the later divergence of Rab and Ras/Rho proteins (Jekely, 2003). Since modern Sar1 and Arf proteins are most strongly associated with regulation of coat proteins, and vesicular trafficking from the ER and the Golgi respectively, this may indicate their ancestors evolved alongside the progenitors of these organelles (Aridor *et al.*, 2001; Jekely, 2003; D'Souza-Schorey and Chavrier, 2006).

The function of the primitive exocytotic secretion may have initially been in the release of lytic enzymes for the extracellular digestion of neighbouring cells in dense bacterial colonies, and systems may have developed for transport and targeting of secretory vesicles to specific sites (Jekely,

2003). While ancestral endocytotic pathways may have had a role in nutrient uptake, the more complex systems required to orchestrate identification, active engulfment, and intracellular digestion of prey through phagocytosis, may have evolved later (Jekely, 2003; Stuart and Ezekowitz, 2005; de Duve, 2007). Indeed, regulated focal exocytotic membrane insertion is directly involved in phagocytosis by macrophages, and it is possible that this involvement dates back to phagocytosis by a phagotrophic LCEA (Cavalier-Smith, 2002; Chieregatti and Meldolesi, 2005).

1.2 Regulated exocytosis

As discussed above, it is possible that specialised exocytotic pathways evolved even prior to the emergence of eukaryotic life. Today, exocytosis occurs in all eukaryotic cells capable of growth and division, because the fusion of exocytotic vesicles with the plasma membrane is essential for the addition of lipid and membrane to this organelle, its maintenance, and its expansion (Burgoyne and Morgan, 2003). It is usual to distinguish constitutive exocytosis, which may serve this purpose, and which is common to all eukaryotes, and regulated exocytosis, which occurs only in response to specific stimuli (Burgoyne and Morgan, 2003).

The wide functional potential of regulated exocytosis is reflected by the considerable variety of its roles (Burgoyne and Morgan, 2003; Chieregatti

and Meldolesi, 2005). Examples of non-secretory regulated exocytosis include that underlying the translocation of GLUT4 transporters to the plasma membranes of adipocytes in response to signalling downstream of insulin receptor activation, and that underlying cytokinesis (Danilchik *et al.*, 2003; Watson *et al.*, 2004). Examples of regulated secretory exocytosis include that underlying release of insulin by pancreatic β-cells, and acrosomal exocytosis in sperm (Ramalho-Santos *et al.*, 2002; Rutter and Hill, 2006). The contrasting roles and dramatic functional specialisation of different forms of regulated exocytosis are illustrated well at the synapse. At a typical presynaptic terminal, the major exocytotic pathway is highly specialised for the very rapid release of the contents of small synaptic vesicles in response calcium influx (Wojcik and Brose, 2007). At the postsynaptic terminal, raised calcium can lead to exocytotic insertion of receptors into the plasma membrane. For example, postsynaptic insertion of the AMPA receptor subunit GluR1 is stimulated during long-term potentiation (LTP) in a process requiring the calcium-Calmodulin-activated kinase CaMKII (Hayashi *et al.*, 2000).

The pathway of regulated exocytosis is often described as a multi-step process, in which following their biogenesis, vesicles are sequentially trafficked, tethered and docked, prior to their fusion with the target membrane in response to a specific trigger. Since this study is focused on regulated secretion, and in particular on the function and regulation of Rab protein isoforms found to associate with secretory granules and synaptic

vesicles, particular emphasis will be placed on these organelles in discussion of each step.

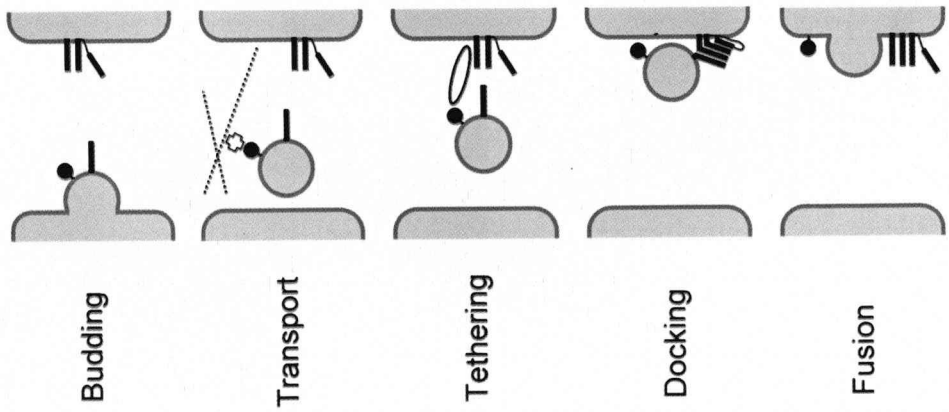


Figure 1.1. Steps in membrane traffic. A vesicle first buds from its donor compartment and is then transported in a cytoskeleton-dependent manner to its target membrane at which it is sequentially tethered and docked, and may then fuse.

1.2.1 Vesicle biogenesis

Protein constituents of secretory granules and synaptic vesicles are synthesised at ribosomes bound to the ER, and translocated into the lumen of this organelle through protein-conducting channels (Rapoport, 1992). Within the ER, chaperone molecules may aid the correct folding of nascent polypeptide, and enzymes may catalyse its initial post-translational modification by glycosylation (Hammond and Helenius, 1995). In higher eukaryotes, protein may exit the ER at ER exit sites (ERES) via vesicles formed through COPII coat-driven budding, and post-ER sorting may occur following fusion of these vesicles with ER-Golgi intermediate compartments (ERGICs)(Appenzeller-Herzog and Hauri, 2006). The anterograde transport of secretory protein to the cis-Golgi within ERGIC-derived carriers is thought to occur in a microtubule-dependent manner (Appenzeller-Herzog and Hauri, 2006).

In the Golgi, protein may undergo further post-translational modification and sorting as it progresses through the ordered compartments of this organelle through cisternal or vesicular transport (Glick, 2000). The exit of proteins from the Golgi occurs at the trans-Golgi network (TGN). It is thought that the constituents of secretory granules aggregate at the TGN, possibly initiating granule formation and budding (Burgoyne and Morgan, 2003; Borgonovo *et al.*, 2006). Once released from the TGN, immature secretory granules may be rapidly transported to a localisation consistent with their particular function. Missorted material may be removed from these granules in a Clathrin-dependent manner, and they may undergo homotypic fusion in the course of their maturation (Tooze *et al.*, 1991; Dittie *et al.*, 1997; Rudolf *et al.*, 2001). In contrast to this, the transport vesicle(s) carrying constituents of synaptic and synaptic-like vesicles from the TGN are not thought to constitute their precursors. Instead, it is thought that these organelles may be later derived from either early endosomes or plasma membrane (Hannah *et al.*, 1999). Since synaptic vesicles may take up their neurotransmitter cargo directly from the cytosol via integral membrane transporters, they may be formed de novo in the same manner as they are recycled (Hannah *et al.*, 1999).

1.2.2 Vesicle trafficking

In common with vesicles in several other membrane trafficking pathways, secretory granules and synaptic vesicles may be directed to their target membrane along microtubules and actin filaments, and these modes of transport may be sequential and coordinated (Goodson *et al.*, 1997; Rudolf

et al., 2001). In many cases, it is thought that Rab proteins associated with particular types of vesicle may specify the route taken through the cytoskeleton by direct or indirect interaction with appropriate motor proteins (Jordens *et al.*, 2005). In anterograde secretory granule trafficking, kinesin-driven transport along microtubules can deliver granules to F-actin-rich regions of cells, while myosins can then facilitate their recruitment to, and regulate their movement within these regions (Rose *et al.*, 2002).

In PC12 cells, it is found that secretory granules within the cortical actin network are segregated into differently mobile populations during maturation, with a large proportion characterised as becoming immobile (Rudolf *et al.*, 2001). It is likely that interactions with cortical actin underlie this apparent system of granule storage, since overexpression of the Rab27A effector MyRIP, which links this protein to actin via MyosinVIIa, further reduces granule mobility, and does so in a manner sensitive to changes in actin stability (Desnos *et al.*, 2003). Conversely, where Rab27A, its effector Melanophilin, or the motor protein MyosinVa are absent from melanocytes, melanosomes are found to cluster in central regions of cells and are also excluded from the actin-rich dendrites of these cells (Wu *et al.*, 1998; Wilson *et al.*, 2000; Provance *et al.*, 2002).

In that secretory granules can be sequestered in F-actin networks in a subcellular localisation appropriate to their function, while they may also move within these networks, it may be suggested that their progress

towards the plasma membrane is precisely choreographed by factors that define their cytoskeletal interactions. In support of this view, movement of granules located close to the plasma membrane in adrenal chromaffin and PC12 cells, is not only reduced by pharmacological stabilisation of actin, but also by its destabilisation (Lang *et al.*, 2000; Oheim and Stuhmer, 2000). Furthermore, where mobile granules are seen to approach the plasma membrane in these cells, they often show a characteristic linear trajectory, indicative of their motor-driven trafficking along single actin filaments (Allersma *et al.*, 2006). In adrenal chromaffin cells, it has been shown that granules approaching the plasma membrane in this manner can contribute more to secretory responses than those already membrane-associated prior to stimulation, underlining the regulatory importance of this active trafficking (Han, 1999; Allersma *et al.*, 2006).

F-actin networks should not be seen as static substrates for the factors directing granule traffic, since actin filaments are subject to continual, polarised, assembly and disassembly, and their organisation is continually subject to local and global remodelling (Lanzetti, 2007). Further, these processes have broad significance for granule trafficking, because actin stabilising and destabilising drugs respectively reduce and enhance secretory responses in a wide range of secretory cell types (Burgoyne and Morgan, 2003). Indeed, in many of these cells, physiological stimuli have been shown to cause actin filament disassembly (Cheek and Burgoyne, 1992). In concert with the other systems involved, this may serve to

promote exocytosis and replenishment of secretory granules at exocytotic sites (Burgoyne and Morgan, 2003).

1.2.3 Tethering

Vesicles or granules attached to the plasma membrane are said to be either 'tethered' or 'docked'. In initial studies with electron microscopy, it was found that among a population synaptic vesicles located within 10nm of the presynaptic membrane, a fraction appear to remain associated in isolated membrane preparations, and these may be termed 'morphologically docked' (Burgoyne and Morgan, 2003). 'Morphologically docked' vesicles were at first thought to be membrane-associated as a result of the engagement of the core machinery of membrane fusion, the SNAP-receptor (SNARE) proteins (Sollner *et al.*, 1993). Therefore, the term 'tethered' was applied to vesicles closely associated to membranes through protein-protein interactions occurring prior to this engagement. However, protein-protein interactions mediating 'tethering', as defined in this way, have been found to be extremely diverse, ranging from those linking vesicles or granules to membrane-associated actin filaments at points very close to the plasma membrane, to those mediating much tighter vesicle-membrane association. In fact, 'tethered' vesicles may be morphologically indistinguishable from vesicles associated with the membrane via SNAREs (Burgoyne and Morgan, 2003).

A well characterised tethering complex in the exocytotic pathway is the exocyst, an eight-subunit complex that multimerises to form a quatrefoil

(Novick *et al.*, 1980; TerBush *et al.*, 1996; Munson and Novick, 2006). In yeast, the exocyst is localised to sites of polarised exocytosis, and mutations in its subunits can lead to defective exocytosis and accumulation of secretory vesicles (TerBush *et al.*, 1996). The protein components of the exocyst are conserved in higher eukaryotes, and are present at secretory sites in various cell types (Kee *et al.*, 1997). In one model of mammalian exocyst function, exocyst components are suggested to be recycled between the TGN and plasma membrane, and therefore to have a multifunctional role in targeted vesicle trafficking. Treatment of epithelial-like NRK-52E cells with antibodies against epitopes of the exocyst component Sec6 specifically accessible at the TGN and the plasma membrane, was found to cause retention of exocytotic vesicles at a perinuclear region and within the cytosol and cortical areas of cells respectively (Yeaman *et al.*, 2001). This data suggests that the exocyst is required for vesicle trafficking from the TGN, while it may also be required in their tethering prior to fusion, since the experiments showed reduced insertion of a fluorescent marker protein into the plasma membrane (Yeaman *et al.*, 2001).

In mammalian neurones, the exocyst is localised to areas of neurite outgrowth and developing synapses (Hazuka *et al.*, 1999). Further, it has been found that NGF-stimulation of PC12 cells leads to translocation of the exocyst to nascent neurites, while overexpression of dominant negative exocyst component sec10 impairs their development (Vega and Hsu, 2001). In Sec5 null Drosophila neurones, growth of neuromuscular

junctions was arrested on the depletion of maternally provided Sec5, and protein trafficking to the plasma membrane was reduced. Furthermore, the traffic of a GFP tagged version of the synaptic vesicle protein Synaptotagmin to synaptic boutons was dramatically impaired (Murthy *et al.*, 2003). Despite this however, synaptic transmission at single boutons was normal, and in other cell types such as PC12 cells, acute disruption of exocyst assembly was not found to affect calcium-triggered secretory responses (Murthy *et al.*, 2003; Wang *et al.*, 2004).

It has been demonstrated in MDCK cells that exocyst disruption can differentially affect the trafficking and exocytosis of different subsets of exocytotic vesicle (Grindstaff *et al.*, 1998). Therefore, in the Drosophila neurones discussed above, it may function in the targeting of transport vesicles carrying Synaptotagmin, but not in that of synaptic vesicles, which as previously discussed, may be locally synthesised at endosomal or plasma membranes (Hannah *et al.*, 1999). Similarly, its involvement in the trafficking and tethering of secretory granules may differ according to the type of granule involved, while evidence suggests that here, this involvement may also differ according the mode by which exocytosis is regulated. It was shown in one study that while the calcium-triggered secretory responses of PC12 cells were not affected by exocyst disruption, those triggered with the non-hydrolysable GTP analogue, GppNHp, were reduced (Wang *et al.*, 2004). In contrast, another study showed that exocyst disruption in the MIN6 pancreatic β-cell line led to a reduction in numbers of secretory granules close to the plasma membrane, and

corresponding reductions in secretory responses to several different stimuli (Tsuboi *et al.*, 2005).

The tethering of synaptic vesicles at the presynaptic nerve terminal may best be understood as one function of the highly ordered arrays of multimolecular complexes that form the active zone (Zhai and Bellen, 2004). The cytomatrix underlying the plasma membrane at the active zone is highly electron dense, and is comprised of various cytoskeletal proteins arrayed in a web like pattern, and scaffolding proteins which link this structure to ion channels and the fusion machinery (Phillips *et al.*, 2001). While many proteins within this matrix may have a tethering function, this is most easily ascribed to electron dense structures projecting from it, known as synaptic ribbons (Prescott and Zenisek, 2005). Large synaptic ribbons were first identified at sensory synapses, where they can adopt various forms, and may extend far from the plasma membrane (Prescott and Zenisek, 2005). These 'ribbons' sequester synaptic vesicles in the vicinity of the active zone, and are suggested to function where sustained or high intensity neurotransmitter output is required (Lenzi *et al.*, 2002). Less prominent projections in mammalian central synapses are very likely to fulfil a similar purpose (Phillips *et al.*, 2001; Zhai and Bellen, 2004).

In secretory cells where neither the exocyst nor the neurone-specific components of the active zone limit exocytosis, the molecular identity of tethering proteins is less well characterised. However, TIRF microscopy has been used to directly visualise what may be the tethering of secretory

granules in the adrenal chromaffin cell (Toonen *et al.*, 2006). Because Munc18-1 is thought to be required for efficient assembly of exocytotic SNARE complexes in chromaffin cells, any association between secretory granules and the plasma membrane in the absence of this protein is likely to reflect the activity of a tethering mechanism (Voets *et al.*, 2001b; Zilly *et al.*, 2006). In Munc18-1-deficient cells, granule density close to the plasma membrane is reported to be reduced (Voets *et al.*, 2001b). However, when the movements of labeled granules in these cells were recorded using TIRFM, it was found that numbers of granules transiently entering the evanescent field in the course of experiments were lower than those in controls (Toonen *et al.*, 2006). Thus, reduced granule density at the membrane may result from impaired granule delivery, rather than tethering or docking deficits. In fact, the numbers of granules entering the evanescent field and associating with the plasma membrane for periods of time longer than ~5 seconds were comparable between control and Munc18-1-deficient cells (Toonen *et al.*, 2006). It may therefore be suggested that such granules are tethered in a Munc18-1-independent manner.

While the numbers of granules adopting a sustained interaction with the plasma membrane in the course of the experiments described was low, the authors provided additional evidence for the above suggestion. Where the average responsiveness of granules to movement of the plasma membrane was calculated, it was reduced four-fold in the Munc18-1-deficient cells, indicating that these granules were less closely membrane-

associated (Toonen *et al.*, 2006). The implication of this finding is that tethered granules may become more closely membrane-associated through the action of Munc18-1. Interestingly, since data was derived from granules associating with, and then dissociating from the plasma membrane, this finding also suggests that under resting conditions, Munc18-1-dependent tight membrane-association is reversible. Further, the similar distribution of granule-membrane association times of between 5 and 100 seconds in the data sets from the control and Munc18-1-deficient cells may suggest that Munc18-1 expression, at least at endogenous levels, has minimal effects on these times, though the authors' interpretation of this data differs (Toonen *et al.*, 2006).

1.2.4 Docking

As discussed above, the term 'docked' may be used to describe associations between vesicular or granular, and plasma membranes, that involve interactions between combinations of SNARE proteins required in the their subsequent fusion. In this section, discussion will focus on these interactions, and on the suggested role of the SM proteins in their promotion.

36 SNAP-receptor (SNARE) proteins, characterised by a heptad repeat SNARE motif, are identified in the human genome (Jahn and Scheller, 2006). These proteins may be denoted v-SNAREs or t-SNAREs according to their presence on the vesicular or target membrane, and are thought to drive membrane fusion by formation of stable, coiled-coil, heteroligomeric

complexes comprised of four parallel α-helices (Hanson *et al.*, 1997a; Sutton *et al.*, 1998). Each α-helix corresponds to a particular SNARE motif, and typically, but not exclusively, one is contributed by a v-SNARE, while the remaining three are contributed by t-SNAREs (Jahn and Scheller, 2006). Amino acid side chains interacting within the centre of the coiled-coil bundle are usually hydrophobic, while conserved hydrophilic residues in each α-helix contribute to interactions at a central, 'zero', layer that is shielded within each complex (Fasshauer *et al.*, 1998). In each SNARE complex, one of these residues is arginine (R), and three are glutamine (Q). Thus, in order to classify SNARE proteins in a manner that allows for clarity in the discussion of atypical and homotypic fusion events, each contributory SNARE motif may denoted R, Q_a, Q_b or Q_c, and each SNARE protein may be denoted an R-SNARE or a Q-SNARE (Fasshauer *et al.*, 1998; Jahn and Scheller, 2006).

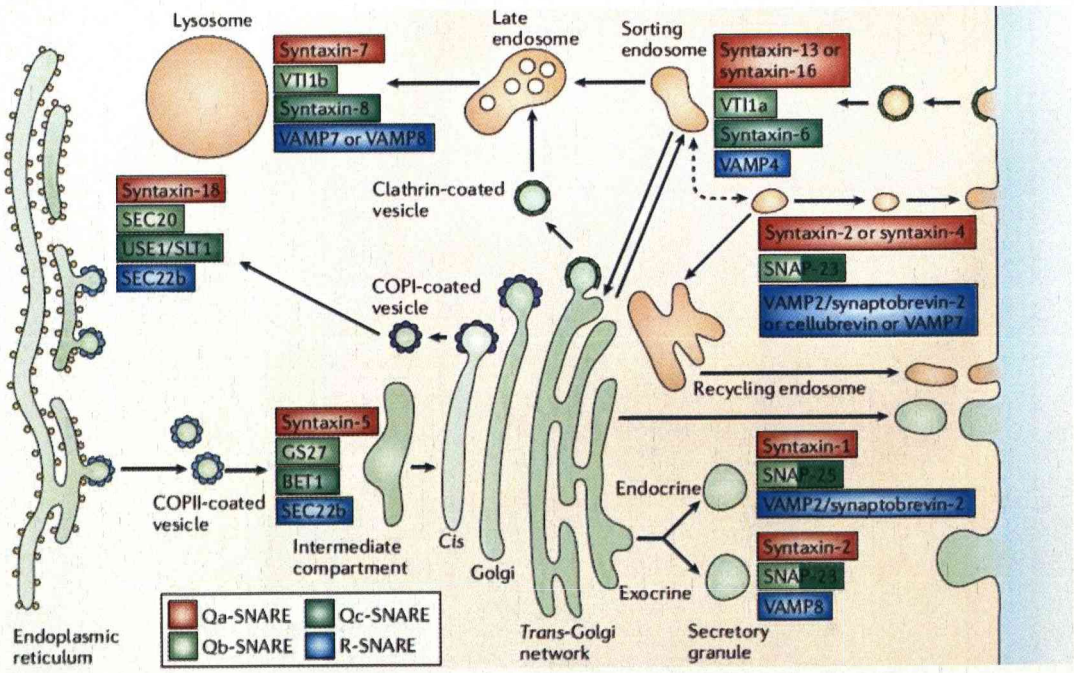


Figure 1.2. SNARE protein combinations with identified function in traffic between specific membrane compartments in mammalian cells. SNARE proteins contributing Q_a, Q_b, Q_c and R helices to each complex are indicated. Diagram is taken from Jahn and Scheller, 2006.

Particular combinations of SNARE proteins contribute to the specificity of fusion between specific vesicular and target membranes, while the extent of this contribution is a subject of debate (Burgoyne and Morgan, 2003; Jahn and Scheller, 2006). The best-characterised SNARE complex associated with exocytotic fusion is that formed by the R-SNARE vesicle-associated membrane protein-2 (VAMP2/Synaptobrevin), and the Q-SNAREs Syntaxin1A and synaptosomal associated protein of 25kDa (SNAP-25)(Sutton *et al.*, 1998; Ernst and Brunger, 2003). VAMP and Syntaxin are single pass transmembrane proteins, and respectively contribute R and Q_a helices to the complex. SNAP-25 is membrane-associated as a result of its palmitoylation, and contributes the remaining two helices Q_{bc} (Sutton *et al.*, 1998; Ernst and Brunger, 2003).

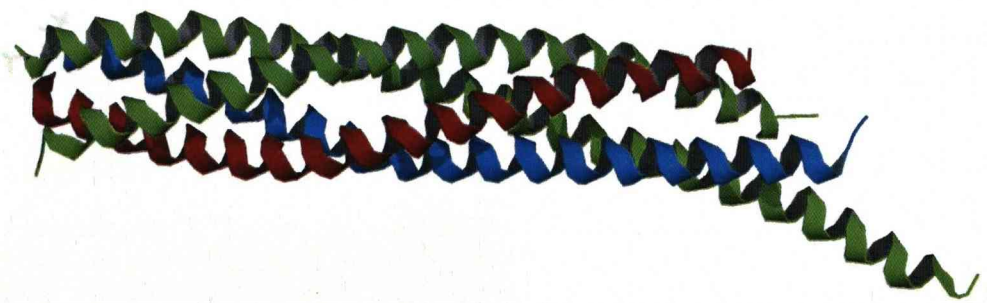


Figure 1.3. Crystal structure of a truncated form of the assembled neuronal SNARE complex. Syntaxin1A, SNAP-25 and VAMP2 are shown in blue, green and red respectively. Molecular coordinates taken from Ernst and Brunger, 2003 (RCSB PDB Structure ID 1N7S).

It has been shown that the assembled neuronal SNARE complex is highly stable, and resistant to proteolytic cleavage and SDS denaturation, and so its assembly is unlikely to be readily reversible (Fasshauer, 2003). However, several lines of evidence point to the suggestion that vesicular VAMP, and Syntaxin and SNAP-25 on the plasma membrane, may form only a partially assembled SNARE complex where granules are 'docked'

(Burgoyne and Morgan, 2003; Jahn and Scheller, 2006). It has been suggested that antibodies unlikely, or unable to bind to the assembled SNARE complex in vivo are nonetheless capable of inhibiting exocytosis of the most rapidly released secretory granules in adrenal chromaffin cells (Xu *et al.*, 1999b). Further, it has been shown that SNARE complexes are likely to assemble in an N- to C-terminal manner, with the N-terminal, membrane distal end, able to assemble independently of the C-terminal, membrane proximal end (Pobbati *et al.*, 2006; Sorensen *et al.*, 2006). Where mutations likely to affect assembly of the final membrane proximal layer of the complex were made, there was a reduction in the fast component of chromaffin cell secretory responses, but not their final magnitude, as judged by capacitance (Sorensen *et al.*, 2006). This is consistent with a reduced rate in the relatively fast formation of fusogenic SNARE complexes in vivo, in the absence of any reduction in the slower formation of 'intermediate', non-fusogenic complexes. Where mutations likely to affect assembly of the middle layers of the complex were made, there was a reduction in the rate and magnitude of secretory responses (Sorensen *et al.*, 2006). This is consistent with a reduction in both fast fusogenic SNARE complex formation, and also in slow 'intermediate' SNARE complex formation.

As discussed in 'Tethering', above, TIRF microscopy appears to show that in the presence of Munc18-1, secretory granules can form closer, albeit reversible, associations with the plasma membrane in chromaffin cells (Toonen *et al.*, 2006). This would be consistent with its promotion of the

formation of SNARE complex intermediates and 'docking', and evidence for this suggestion has recently been presented. It has been reported that VAMP is most readily incorporated into SNARE complexes through interaction with a Q_{abc} 'acceptor' complex containing Syntaxin and SNAP-25 (Pobbati *et al.*, 2006). However, it is thought that in vitro, such complexes are formed only transiently because a second Syntaxin may be incorporated into such complexes to form a Q_{aabc} complex, and VAMP cannot displace this additional Syntaxin (Zhang *et al.*, 2002). Indeed, in in vitro fusion experiments, membranes are routinely cooled to 4°C ostensibly in order to maximise levels of VAMP 'acceptors' (Schaub *et al.*, 2006; Shen *et al.*, 2007). In a recent study, it was shown that Munc18-1 could interact with Syntaxin on membranes isolated from PC12 cells, while it could be displaced by soluble recombinant VAMP, only in the presence of endogenous SNAP-25 (Zilly *et al.*, 2006). This suggested that Munc18-1 might interact with and stabilise the binary Syntaxin/SNAP-25 'acceptor' complex, and such an interaction was subsequently demonstrated in vitro when Syntaxin was mutated to adopt an 'open' conformation (Rickman *et al.*, 2007). Further, data from conventional confocal microscopy, as well as fluorescence lifetime imaging experiments, is consistent with the existence of these 'acceptor' complexes at the plasma membrane in live cells (Rickman *et al.*, 2007). The dramatic acceleration of in vitro liposome fusion seen where Munc18-1 was added to liposomes containing the SNARE complex components supports the suggestion that Munc18-1 is crucial for efficient SNARE complex formation (Shen *et al.*, 2007). The

absence of neurotransmission in Munc18-1 knockout mice also supports this suggestion (Verhage *et al.*, 2000).

1.2.5 Priming

The term 'priming' has been used to describe various steps in the secretory and neurosecretory pathways (Burgoyne and Morgan, 2003). Originally, it was used to describe an ATP-dependent process(es) required to maintain exocytotic responses in secretory cells (Holz *et al.*, 1989). However, it also became used to describe a theoretical mechanism underlying the observation that a fraction of the synaptic vesicles at an active zone are responsive to a given action potential and undergo fusion, while their neighbours do not (Goda and Sudhof, 1997). There are diverse range of ATP-dependent processes thought to maintain secretory responses (Burgoyne and Morgan, 2003). Further, it has been suggested that 'docking' and 'priming of synaptic vesicles may not be mechanistically distinct (Wojcik and Brose, 2007). Therefore, discussion here will be limited to the well-characterised, ATP-dependent role of N-ethylmaleimide-sensitive fusion protein (NSF) and its essential cofactor α-soluble NSF attachment protein (α-SNAP) in exocytosis.

Where v- and t-SNAREs on apposing membranes assemble, the resulting complex is known as a trans-SNARE. Where such complexes are present on a single membrane, following SNARE-mediated fusion for example, they are referred to as cis-SNAREs. Because of the very high stability of cis-SNAREs, the ATP-dependent machinery of NSF and α-SNAP is

required for their disassembly and subsequent recycling (May *et al.*, 2001). These proteins are not thought to disassemble trans-SNARE complexes, however, as they operate as components in a large multimeric complex to which trans-SNAREs are likely to be inaccessible. NSF was first discovered through its ability to reconstitute N-ethylmaleimide-blocked intra-Golgi traffic in vitro (Block *et al.*, 1988). It was found to possess two ATPase domains, one a functional ATPase, and the other involved in formation of a ring-like homohexamer (Hanson *et al.*, 1997b; Furst *et al.*, 2003). Two such homohexameric complexes are thought to associate in an antiparallel manner to form a barrel-like structure, while this complex associates with cis-SNAREs via molecules of α-SNAP (May *et al.*, 2001; Furst *et al.*, 2003). α-SNAP aligns the SNARE complexes with the central 'pore' of the structure, stimulates the ATPase activity of the NSF, and ATP-hydrolysis drives cis-SNARE disassembly, possibly through hydration of the SNARE zero layer (Scales *et al.*, 2001).

Despite the likely specificity of NSF and α-SNAP for cis-SNAREs, there is strong experimental evidence to suggest that they serve a pre-fusion as well as a post-fusion role. Introduction of recombinant α-SNAP into adrenal chromaffin cells was found to proportionately increase the magnitude of the fast and sustained components of their secretory response to elevated calcium (Xu *et al.*, 1999a). Therefore, it is likely that NSF/α-SNAP can in some way increase the probability that any given granule will undergo exocytosis in these cells. Inhibition of NSF in the same system using NEM was found to reduce the sustained component of secretory responses to a

greater extent than their fast component (Xu *et al.*, 1999a). This indicates that NSF/α-SNAP may act to increase the probability of some event upstream of granule fusion rather than the fusion event itself. In view of the of the characterised activity of NSF/α-SNAP, both of these findings are compatible with the suggestion that its continual disassembly of cis-SNARE complexes dynamically determines levels of trans-SNARE complex formation (Xu *et al.*, 1999a; Burgoyne and Morgan, 2003).

How may the suggested function of NSF/α-SNAP be incorporated into a model for the function of the exocytotic machinery already discussed? It has been suggested that vesicular VAMP may be incorporated into cis-SNAREs, and that these must be disassembled prior to the formation of trans-SNAREs (Xu *et al.*, 1999a; Burgoyne and Morgan, 2003). It is also plausible that NSF/α-SNAP disassembles 'functionally inert' plasma membrane Syntaxin/SNAP-25 complexes, and thereby promotes formation of the Munc18/Syntaxin/SNAP-25 Q_{abc} complexes that serve as the 'acceptors' for the vesicular VAMP (Jahn and Scheller, 2006). This would be consistent with the suggestion that Q_{abc} SNAREs are required for Munc18-1 to promote membrane fusion in vitro, and with the report that Munc18-1 gradually dissociates from isolated plasma membranes, and so may slow but not prevent Q_{aabc}-SNARE formation in vivo (Zilly *et al.*, 2006; Shen *et al.*, 2007).

If, as implied above, levels of 'free VAMP' and/or 'acceptor complex' determine the magnitude of secretory responses, then this would support

the previous suggestion that the probability of triggered exocytosis is directly governed by the extent of 'intermediate' trans-SNARE formation (Sorensen *et al.*, 2006). If this suggestion is accepted, however, the finding that introduction of α-SNAP into cells caused proportionate increases in the fast and the sustained components of their exocytotic responses must also be explained in these terms. One possibility is that this finding reflects proportionate increases in trans-SNARE formation at granules tethered prior to stimulation, and at granules that become tethered during stimulation. This interpretation might support previous suggestions made here on the basis of TIRF data, that tethering governs granule-plasma membrane association, while trans-SNARE formation is reversible (Toonen *et al.*, 2006)(see 1.2.3 'tethering' above). If trans-SNARE formation could affect membrane-association, or if it were irreversible, it is likely that increased trans-SNARE assembly would have a greater effect on the fast component of responses than on their sustained component.

The effects of NSF inhibition on secretory responses are similar to those produced where SNARE proteins were mutated in a manner proposed to affect 'intermediate' trans-SNARE assembly (Xu *et al.*, 1999a; Sorensen *et al.*, 2006). This further supports the suggestion that NSF/α-SNAP activity determines the cellular levels of VAMP and/or 'acceptor' complex, which then determine the likelihood of 'intermediate' trans-SNARE formation, subsequent 'fusogenic' trans-SNARE formation and exocytosis (Xu *et al.*, 1999a; Zilly *et al.*, 2006). However, the finding that NSF-inhibition disproportionately affects the sustained component of exocytotic

responses may at first appear to suggest that trans-SNAREs formed prior to stimulation are resistant to the effects of this treatment. Since this explanation would indicate that trans-SNAREs could be formed stably rather than reversibly, it might not be consistent with those suggested for the other findings discussed here.

An alternative explanation for the apparently selective effects of NSF-inhibition might be that prior to stimulation, it has only a small effect on the levels of 'free VAMP' and/or 'acceptor complexes' present in cells. While these levels may be raised rapidly by increased NSF/α-SNAP activity, reduced NSF/α-SNAP activity may not produce a correspondingly rapid fall. The depletion of 'acceptor complexes', for example, might depend on the slow dissociation of Munc18 (Zilly *et al.*, 2006). In this situation, the initial phase of a given secretory response may be relatively unaffected by NSF-inhibition, with its effect becoming more pronounced over time. The results of experiments in which NEM-treated cells were stimulated twice may support this idea. It was found that in NEM-treated cells, secretory responses to the second stimulation were much more dramatically reduced compared to controls, and that the rapid, as well as the sustained release was affected (Xu *et al.*, 1999a). Thus, the depletion of 'free VAMP' and/or 'acceptor complex' levels during the first response must have strongly compounded the effects of NEM on the second.

In conclusion, it is evident that NSF/α-SNAP activity can determine both the size of exocytotic responses, and the capacity of the exocytotic

systems to recover following use. It is likely that the individual components of the secretory machinery are subject to continual change and turnover, while the ATP-dependent 'Priming' reactions catalysed by the NSF/α-SNAP complex maintain the function of the secretory pathway as a whole.

1.2.6 Triggering

It is the 'triggering' step in exocytosis that distinguishes regulated, from constitutive exocytotic pathways. A wide variety of extracellular stimuli can evoke exocytotic responses, while defined exocytotic triggers are a small group of second messengers including cAMP, GTP and calcium. Elevated cAMP can modulate exocytosis in many cell types, while it can trigger exocytosis in cells including pancreatic β-cells and pituitary cells, and may be the primary trigger in parotid acinar cells and submandibular gland cells (Ashby and Tepikin, 2002; Burgoyne and Morgan, 2003). At least in parotid acinar cells, the nucleotide must activate protein kinase A (PKA) in order to trigger an exocytotic response (Fujita-Yoshigaki *et al.*, 1999). Elevated GTP can also trigger exocytosis in a range of cell types including adrenal chromaffin cells and some neurones, although it has not been identified as the primary trigger for exocytosis in these cells (Burgoyne and Morgan, 2003). While a large number of GTP-binding proteins have characterised roles in exocytosis, it has recently been reported that Ral proteins act as GTP sensors in GTP-dependent secretion (Vitale *et al.*, 2005; Li *et al.*, 2007). Calcium is by far the most widespread trigger for exocytosis, and is the primary trigger in neurones, PC12 cells, adrenal chromaffin cells and many other cell types (Burgoyne and Morgan, 2003;

Wojcik and Brose, 2007). Therefore, further discussion here will be restricted to calcium-triggered exocytosis.

Although calcium is a trigger for regulated exocytosis in diverse cell types, the source of this 'calcium-trigger' can vary (Burgoyne and Morgan, 2003). In neuronal cells, and other cell types including adrenal chromaffin cells, calcium influx through voltage-gated channels precipitates the exocytotic response, while in pancreatic acinar cells, for example, it is triggered by the calcium release from internal stores (Ashby and Tepikin, 2002; Wojcik and Brose, 2007). In some other cell types such as pituitary corticotrophs, a combination of calcium influx and release from stores is required to evoke a maximal response (Burgoyne and Morgan, 2003).

Not only may the 'calcium-trigger' take various forms, it is also known to act at a very wide range of proteins known to be involved in the regulation of the exocytotic pathways. These include EF-hand domain-containing calcium-binding proteins such as Calmodulin and the neuronal calcium sensor (NCS) proteins, as well as C2 domain-containing calcium-dependent phospholipid-binding proteins including such as protein kinase C (PKC), and various Rab effector proteins (Fukuda, 2005; Morgan *et al.*, 2005; Burgoyne, 2007). However, it is the C2 domain-containing protein Synaptotagmin that is most widely regarded as the major transducer of the calcium trigger, and it is this protein that discussion will focus on.

The Synaptotagmins are a group of transmembrane proteins each carrying two C2 domains denoted C2A and C2B (Sutton *et al.*, 1995; Sutton, 1999; Yoshihara and Montana, 2004). These domains may function in calcium-dependent interactions with phospholipids or in protein-protein interactions (Nalefski and Falke, 1996). Evidence that Synaptotagmin is important in regulated exocytosis includes findings showing that loss of the protein can impair secretion in a number of animal models. C. elegans lacking the Synaptotagmin-encoding gene snt-1 were found to display behaviour, and responses to pharmacological agents, consistent with impaired neurotransmission (Nonet *et al.*, 1993). Such impairment was observed directly in Drosophila lacking a functional syt gene, and in mice lacking functional Synaptotagmin I (Littleton *et al.*, 1993; Geppert *et al.*, 1994b). Evidence that Synaptotagmin functions as a calcium sensor in exocytosis includes the finding that where mouse Synaptotagmin I is mutated to reduce its affinity for calcium, the calcium sensitivity of neurotransmission is reduced to a similar extent (Fernandez-Chacon *et al.*, 2001).

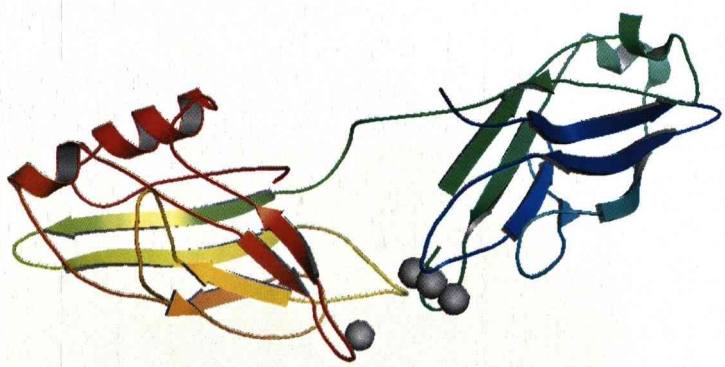


Figure 1.4. Crystal structure of the C2A and C2B domains of Synaptotagmin III. The C2A domain is shown interacting with three metal ions, while the C2B domain is shown interacting with only one. Molecular coordinates taken from Sutton *et al.*, 1999 (RCSB PDB Structure ID 1DQV).

Mammalian Synaptotagmin isoforms, of which there are at least 16, show a range of calcium affinities, and may adopt distinct subcellular localisations. For example, Synaptotagmins I, II and IV predominantly localise to the membranes of synaptic vesicles or secretory granules, and have a lower affinity for calcium than Synaptotagmins III and VII, which predominantly localise to the plasma membrane (Butz *et al.*, 1999; Berton *et al.*, 2000; Sugita *et al.*, 2001; Sugita *et al.*, 2002). It has been suggested that the involvement of different Synaptotagmin isoforms in different exocytotic events might determine the calcium-sensitivity of these events (Sugita *et al.*, 2002). For instance, their involvement might underlie the finding that in neutrophils, progressively greater levels of intracellular calcium are required to trigger exocytosis of secretory vesicles, gelatinase granules, specific granules and azurophil granules (Sengelov *et al.*, 1993). Further, it might underlie the finding that in the neurones and adrenal chromaffin cells of Synaptotagmin I knockout mice, the rapid components of secretory responses are lost, while their sustained component is relatively unaffected (Geppert *et al.*, 1994b; Voets *et al.*, 2001a).

Synaptotagmin I has a comparatively low affinity for calcium, and this is consistent with its functioning as the calcium sensor in rapid responses. In different neurones, the delay between calcium influx and transmitter release is reported to be between 60μs and 300μs (Sabatini and Regehr, 1996; Bollmann *et al.*, 2000). Since the movement of calcium is limited by the rate of its diffusion, this suggests that a calcium sensor must be located close to the point of calcium entry. Indeed, in theoretical analysis

of quantal secretion at the active zone, it is suggested that the probability of synaptic vesicle exocytosis is reduced threefold with a doubling of the distance to the nearest calcium channel from 25nm to 50nm (Bennett *et al.*, 2000). Thus, it is likely that the sensor operates in microdomains of high calcium concentration produced close to an open channel mouth, and so its affinity for the ion may be lower than if it were required to respond to a more global calcium elevation.

The molecular mechanism by which calcium binding to Synaptotagmin I can lead to virtually instant synaptic vesicle exocytosis is the subject of continued debate (Carr and Munson, 2007; Wojcik and Brose, 2007). However, it has been shown to bind to phospholipids, and to C-terminal regions of SNAP-25, in a calcium-dependent manner (Gerona *et al.*, 2000). Its binding to SNAP-25 is very likely to be significant in its function, as it binds to an exposed region of the protein that forms part of the C-terminal end of the $Q_{abc}R$ SNARE complex, and which is important in defining the calcium-dependency of the exocytotic response (Gerona *et al.*, 2000; Sorensen *et al.*, 2006). Further, several recent studies have indicated that its function is related to that of another protein, Complexin (Giraudo *et al.*, 2006; Schaub *et al.*, 2006; Tang *et al.*, 2006).

Complexins I and II are implicated in rapid exocytotic responses in neurones because their absence results in reduced calcium-triggered synchronous neurotransmitter release (Reim *et al.*, 2001; Xue *et al.*, 2007). It is found that their central α-helical domains within these proteins can

bind to assembled SNARE complexes in the 'groove' formed between the Syntaxin and VAMP SNARE motifs (Q_aR), while their function also depends on their adjacent N-terminal domains (Bracher *et al.*, 2002; Chen *et al.*, 2002; Xue *et al.*, 2007). Microinjection of recombinant Complexin into Aplysia neurones inhibits evoked neurotransmitter release, and a similar effect is produced where a VAMP-Complexin fusion protein is expressed in mammalian neurones to raise Complexin concentration at the fusion machinery specifically (Ono *et al.*, 1998; Tang *et al.*, 2006). Similarly, in vitro, the addition of recombinant Complexin to liposome fusion assays, or cell-based fusion assays, is also inhibitory (Giraudo *et al.*, 2006; Schaub *et al.*, 2006). Together, these data indicate that Complexin may function as a 'fusion clamp', limiting the capacity of the SNARE complex to drive membrane fusion.

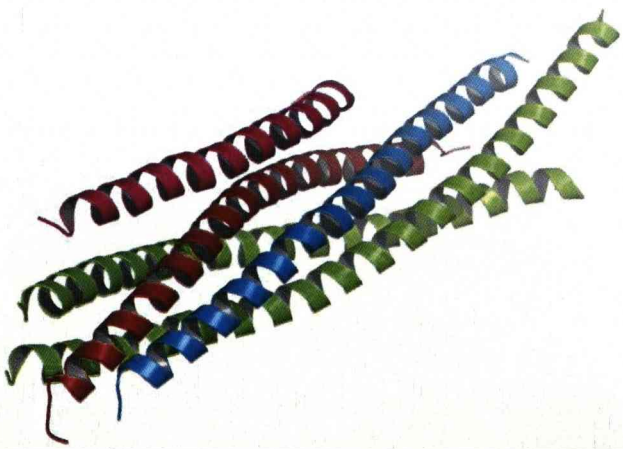


Figure 1.5. Crystal structure of a truncated form of the assembled neuronal SNARE complex bound a Complexin fragment. Complexin (pink) binds in a 'groove' formed between VAMP2 (red) and Syntaxin1A (blue). SNAP-25 is shown in green. Molecular coordinates taken from Chen et al., 2002 (RCSB PDB Structure ID 1KIL).

Given that both Synaptotagmin I and Complexin are required for synchronous neurotransmitter release, it was possible that they function as components of the same triggering machinery, and so a number of studies

were carried out to test this hypothesis. In vitro, and therefore in the absence of other factors, Complexin-blocked SNARE-driven liposome fusion was restored by the addition of calcium and Synaptotagmin I, and in fact proceeded faster than under conditions where both Complexin and Synaptotagmin were absent (Schaub *et al.*, 2006). In other experiments, addition of calcium to a cell based fusion assay containing Complexin and Synaptotagmin I was shown to trigger fusion, while in vitro binding of Complexin and Synaptotagmin I to SNARE complexes was found to be competitive (Giraudo *et al.*, 2006; Tang *et al.*, 2006). Therefore, it appears that calcium binding to Synaptotagmin I triggers rapid secretory responses by lifting a block imposed on the exocytosis of 'docked' synaptic vesicles by the binding of Complexin to trans-SNAREs. An interesting feature of the in vitro liposome fusion data, was that the binding of Complexin to trans-SNAREs appeared to promote hemifusion between the outer leaflets of liposomal membranes (Schaub *et al.*, 2006). This seems to suggest that the protein can hold the rest of the exocytotic machinery at the brink of producing fusion, until calcium acts at Synaptotagmin I to trigger it.

1.2.7 Fusion

As already discussed, it has long been suggested that SNARE proteins, and more specifically trans-SNAREs, are responsible for driving the fusion of phospholipid bilayers in the exocytotic fusion event (Hanson *et al.*, 1997a; Burgoyne and Morgan, 2003). Strong evidence for this suggestion includes findings that some combinations of SNARE proteins can reconstitute liposomal fusion in vitro, and that exocytosis can be blocked

where particular SNARE proteins are absent, or cleaved by highly specific clostridial neurotoxins (McNew *et al.*, 2000; Parlati *et al.*, 2000; Burgoyne and Morgan, 2003). Membrane fusion requires energy in order to overcome the powerful repulsion between the headgroups of apposed bilayers (Kozlovsky and Kozlov, 2002). It has been proposed that this energy is provided by the energetically favourable formation of trans-SNARE complexes, which might locally bring bilayers into close apposition. This proposal was formalised as the 'SNARE Zipper hypothesis' (Hanson *et al.*, 1997a).

It is widely accepted that there is a requirement for SNARE proteins in bringing vesicle/granule and plasma membranes into close apposition (Burgoyne and Morgan, 2003; Jahn and Scheller, 2006). However, in recent years, a number of researchers have questioned the assertion that SNARE complex assembly mediates their final fusion. It is suggested, for example, that Complexin binds to fully assembled trans-SNARE complexes prior to triggering (Giraudo *et al.*, 2006; Schaub *et al.*, 2006; Tang *et al.*, 2006). While it is highly plausible that other factors might mediate, or facilitate the final fusion event, it is, difficult to reconcile notion of complete, metastable, SNARE complex assembly, with the degree of reversibility likely in tethering and docking, as discussed (Xu *et al.*, 1999a; Pobbati *et al.*, 2006; Sorensen *et al.*, 2006; Toonen *et al.*, 2006). Also as discussed, several lines of evidence point to stepwise SNARE assembly, with the final step producing a fusion pore (Pobbati *et al.*, 2006; Sorensen *et al.*, 2006). Most importantly, it is found that mutation of residues in

SNAP-25 likely to affect its interactions with the other SNARE proteins in the final step of trans-SNARE assembly specifically slow down the fast component of the exocytotic response (Sorensen *et al.*, 2006). It is probable, therefore, that the forces driving the transition from 'intermediate' to 'fully-assembled' trans-SNARE also determine the likelihood of fusion.

If trans-SNAREs exist in a partially assembled 'intermediate' state prior to full assembly and membrane fusion as argued above, the nature of this state should be discussed. Further, if Complexin binds to 'intermediate' trans-SNAREs prior to fusion, the finding that its addition to an in vitro fusion assay promotes hemifusion between the outer leaflets of liposomes must also be explained in these terms. To address these points, it may be useful to explore the process of membrane fusion more deeply. The principal paradigm for phospholipid bilayer fusion is known as the 'stalk hypothesis' (Kozlovsky and Kozlov, 2002; Blumenthal *et al.*, 2003). Within this paradigm, the local approach of bilayers, where they are 'pulled together' can lead to transient, extreme membrane deformation and lipid distortion that results in hemifusion and continuity between a contacting monolayer, the 'stalk' (Blumenthal *et al.*, 2003). Constrained, hemifused membranes must adopt the most energetically stable conformation possible, a characteristic 'x' shape in cross section, while phospholipids are still subject to distortion at this intermediate stage (Kozlovsky and Kozlov, 2002). For progression from a hemifused to a fully fused state, it is thought that the contacting 'stalk' must be expanded or 'pulled apart', while it is not known whether this leads to immediate bilayer fusion, or whether

that follows formation of a 'fusion diaphragm' (Kozlovsky *et al.*, 2002; Kozlovsky and Kozlov, 2002).

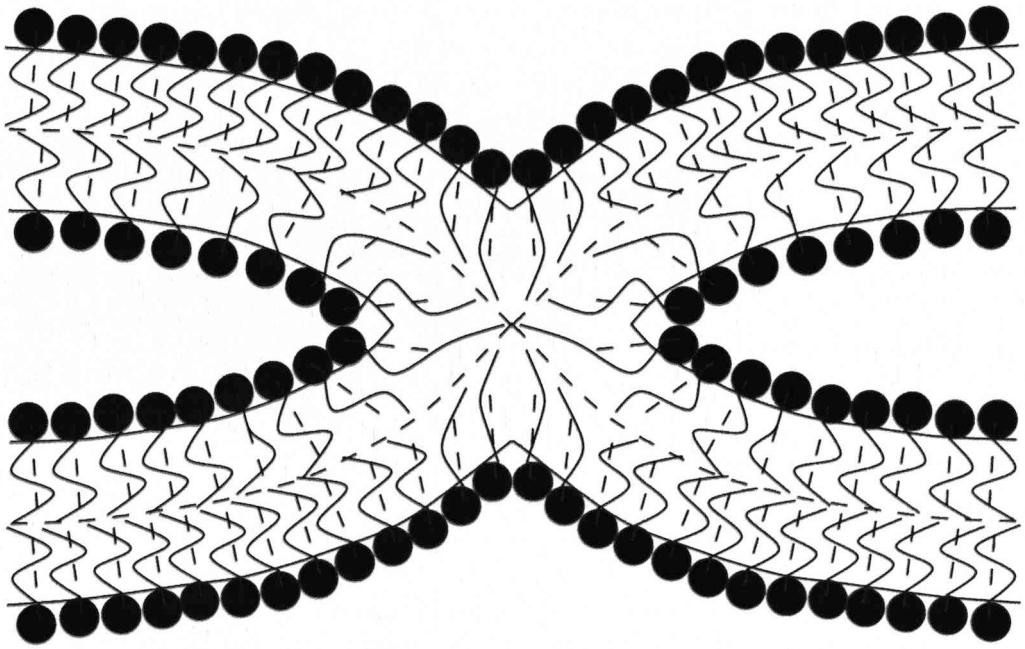


Figure 1.6. Likely structure of the 'stalk' as proposed in the stalk hypothesis of membrane fusion. Diagram shows hemifusion between apposed monolayers of two phospholipid bilayers – the hemifused state is suggested to precede full fusion. Diagram modified from Kozlovsy and Kozlov, 2002

It was argued here earlier that secretory granules tethered at the plasma membrane can become 'docked', reversibly, as a result of 'intermediate' trans-SNARE engagement. Further, it was suggested on the basis of TIRF data that this engagement was reflected in a greater responsiveness of granules to movement of the plasma membrane - a closer association with it (Toonen *et al.*, 2006). According to the paradigm described above, progressively closer membrane association might lead to progressive membrane instability until a relatively stable hemifused state is reached. Therefore, it may be suggested that 'intermediate' trans-SNARE assembly drives membranes towards hemifusion, and complete trans-SNARE

assembly drives full fusion. This seems intuitively likely, since there is evidence for two-step SNARE complex assembly, while the paradigm for membrane fusion suggests that to promote fusion, force may first draw phospholipid bilayers together, and then may draw a phospholipid bilayer apart: two mechanically distinct operations (Kozlovsky *et al.*, 2002; Kozlovsky and Kozlov, 2002). Further there is evidence from a cell-based fusion assay that the minimal SNARE machinery of VAMP, Syntaxin and SNAP-25 can produce stable hemifusion between biological membranes (Giraudo *et al.*, 2005). More subjectively, EM images of the active zone of the frog neuromuscular junction appear to show that the active zone tethering machinery promotes membrane deformation consistent with stable hemifusion between synaptic vesicles and the plasma membrane (Harlow *et al.*, 2001).

To integrate the above model into one in which 'tethering' is the major determinant of membrane-association, and in which 'docking' is reversible, it may be suggested that membrane-associated vesicles or granules are continually at equilibrium between tethered/unfused and docked/hemifused states. In this context, it might be suggested that the finding that Complexin promotes hemifusion between liposomes in vitro reflects its stabilisation of 'intermediate' trans-SNAREs, and its consequent extension of the average hemifusion 'lifetime'. In other words, Complexin might push the equilibrium between tethered/unfused and docked/hemifused states towards the latter.

If it is essential that hemifusion precedes fusion, as suggested in the 'stalk hypothesis', an interesting consequence of the above suggestion would be that the Complexin-bound fusion machinery might promote more rapid exocytosis, though this would of course depend on calcium and Synaptotagmin. Within this model, the Complexin-Synaptotagmin triggering mechanism would define the fast component of exocytotic responses. The sustained component of release would be slower as a result its dependence on the relatively unstable formation of 'intermediate' trans-SNAREs in the absence of Complexin. Consistent with this model, it is found that the sustained phase of exocytotic responses is relatively unaffected in characterised Complexin and Synaptotagmin knockout animals (Geppert *et al.*, 1994b; Reim *et al.*, 2001; Voets *et al.*, 2001a; Xue *et al.*, 2007).

When membranes fuse, a fusion pore is created. The nature of the fusion pore is debated, at one extreme it is proposed to be a channel-like assembly lined by the transmembrane domains of trans-SNAREs, and at the other, to result from the increasing instability of a hypothetical 'fusion diaphragm' with its increasing radius (Kozlovsky *et al.*, 2002; Han *et al.*, 2004; Szule and Coorssen, 2004). It is thought, however, that fusion pores share some of the characteristics of large ion channels or gap junctions (Burgoyne and Morgan, 2003). That is, initial current flow through the pores can be of a defined magnitude, some pores may remain stably open for extended periods, and pore 'flickering' – suggestive of rapid opening and re-closure – has been observed (Fernandez *et al.*, 1984; Wang *et al.*,

2003). Experiments utilising carbon-fibre amperometry have suggested that proteins affecting the kinetics of fusion pore opening and expansion include Munc18 and Synaptotagmin (Fisher *et al.*, 2001; Wang *et al.*, 2003; Wang *et al.*, 2006).

A direct role for Munc18 in fusion is suggested by evidence that it can interact with assembled SNARE complexes (Rickman *et al.*, 2007; Shen *et al.*, 2007). Further, it is found that expression of a Munc18 mutant with reduced affinity for Syntaxin alters kinetics of release from chromaffin granules in a manner consistent with faster opening and closing of the fusion pore (Fisher *et al.*, 2001). Significantly, physiological phosphorylation of Munc18 may have the same effects on its interactions with Syntaxin, and on fusion pore kinetics (Barclay *et al.*, 2003). Like Munc18, Synaptotagmin I can interact with assembled SNARE complexes, and is found to accelerate liposome fusion in vitro (Schaub *et al.*, 2006; Stein *et al.*, 2007). A direct role for the protein in fusion is suggested by evidence that it may be involved in the stabilisation of minimally conductive fusion pores, and their subsequent expansion (Wang *et al.*, 2003; Wang *et al.*, 2006). In this case, these effects may be physiologically significant because they vary with calcium concentration, and Synaptotagmin isoform (Wang *et al.*, 2003; Wang *et al.*, 2006).

If it is accepted that the full assembly of trans-SNAREs drives fusion, it is possible that Munc18 and Synaptotagmin affect this assembly in some way, while it is also possible that they contribute to the fusion process

directly. As their role remains unclear, these possibilities will not be discussed here. However, their possible roles in regulating quantal release and fusion pore kinetics may be physiologically important, and relevant to the discussion of membrane retrieval below.

1.2.8 Retrieval

Full exocytotic fusion may proceed with expansion of the fusion pore leading to incorporation of granular or vesicular membrane into the plasma membrane. In order to maintain the surface area of the plasma membrane, and for the retrieval of some of the granular/vesicular exocytotic machinery, membrane may be recycled through Clathrin-based and/or bulk endocytosis (Royle and Lagnado, 2003). The molecular mechanisms by which these forms of recycling occur are largely distinct from those directly involved in exocytosis, and so their discussion is beyond the scope of this introduction. However, another form of membrane recycling termed 'kiss-and-run' should be discussed (Fesce *et al.*, 1994). In 'kiss-and-run' exo/endocytosis, granular/vesicular membrane is not fully incorporated into the plasma membrane. Instead, granules or vesicles are recycled following the release of their contents or a portion of their contents (An and Zenisek, 2004; Rutter and Tsuboi, 2004).

Evidence for the existence of 'kiss-and-run' comes from a number of sources. Experiments in which amperometry was combined with capacitance recordings showed that catecholamine release from single chromaffin cell granules could coincide with transient increases in

membrane capacitance (Albillos *et al.*, 1997; Ales *et al.*, 1999). This suggests that fusion between granular and plasma membranes can be closely followed by their fission (Burgoyne and Morgan, 2003). In neurones, quantal release is smaller, and so not amenable to this kind of direct approach. However, particularly at low levels of stimulation, fluorescent FM- dyes with different affinities for lipid may be released from vesicles at different rates (Cousin and Robinson, 2000; Stevens and Williams, 2000). This may suggest that kiss-and-run occurs in these cells because parallel destaining could be expected in the absence of transient fusion events. This is however, controversial, and has been disputed in a recent study (Granseth *et al.*, 2006).

Various physiological roles for kiss-and-run might be proposed. One suggestion is that retrieval of granule or vesicle membrane via 'kiss-and-run' might be more energy-efficient than its retrieval via a Clathrin-dependent mechanism. In neurones, 'kiss-and-run' recycling of synaptic vesicles followed by their rapid refilling might also increase the rate at which they are replenished following their depletion (Rizzoli and Jahn, 2007). Another possibility is that 'kiss-and-run' is regulated in order to alter the quantal size of release events, and possibly their character. For example, secretory granules might release only a fraction of their peptide contents through a transient fusion pore, but a greater proportion of any small molecule neurotransmitter content (Burgoyne and Barclay, 2002). It is possible that this might serve to modulate distinct responses at the cells' targets (Burgoyne and Barclay, 2002). In neurones, it has been suggested

that very limited neurotransmitter release through fusion pores with low conductance may be involved in sensitisation of post-synaptic receptors (Choi *et al.*, 2000). Thus, release of the same neurotransmitter from the same cell, but at different levels and under different circumstances, might regulate different post-synaptic processes.

The mechanisms underlying transient fusion are unknown, and indeed it is likely that distinct forms of 'kiss-and-run' exist. It has been shown that expression of specific Munc18 mutants in chromaffin cells, or treatment of these cells with the phorbol ester PMA, can reduce the quantal size of release events, and alter release kinetics (Fisher *et al.*, 2001; Graham *et al.*, 2002; Barclay *et al.*, 2003). These changes in release kinetics have been suggested to correspond to faster opening and closure of fusion pores, and one form of 'kiss-and-run'. Further, they are partially reversed by treatments likely to inhibit the function of the Dynamin fission machinery, suggesting that this machinery may act to 'pinch-off' partially fused granules following a proportion of transient fusion events (Graham *et al.*, 2002). In other studies, it has been suggested that overexpression of particular Synaptotagmin isoforms, or mutation of this protein in PC12 cells, can produce changes in the numbers and duration of amperometric events suggested to correspond to neurotransmitter release through poorly conductive fusion pores (Wang *et al.*, 2003; Wang *et al.*, 2006). The molecular basis for opening and closure of these pores, and that for their expansion, is poorly understood, but it is unlikely to be the same as that for the opening and closure of more conductive pores already described.

1.3 Regulation of regulated exocytosis

In the above discussion of the regulated exocytotic pathway, emphasis has been placed on the past characterisation of the molecular machinery central to each step, and on the dynamic behaviour of this machinery. Given this focus, it should be noted that no attempt has been made to present a complete picture. For instance, no mention has been made of the role of key proteins such as Munc13, which is essential for neurotransmitter release, or the membrane modifications and makeup associated with exocytotic sites and events (Osborne *et al.*, 2006; Wojcik and Brose, 2007). More importantly, however, the regulation and modulation of the exocytotic pathway as a whole has not yet been discussed directly. Multiple factors cooperatively regulate various aspects of each step in the pathway, and so a brief discussion here will explore, in general terms, how exocytotic systems are structured so that secretory responses are maintained and adaptively amenable to regulation.

One important observation is that the granules or vesicles of a given cell are often functionally segregated into 'readily-releasable' and 'reserve' pools. That is, one pool that undergoes exocytosis readily in response to a trigger, and another that is mobilised to replenish this pool as it is depleted. The 'readily-releasable' pool is primarily responsible for defining the size of exocytotic responses, while the 'reserve' pool determines the regenerative capacity of the system. Interestingly, it has been shown in both secretory cells and in neurones that these pools may not be distinguished

morphologically (Han, 1999; Rizzoli and Betz, 2005). Of course, the synaptic vesicles exocytosed within microseconds of calcium influx are those present within a population associated with the active zone, but typically, a morphologically similar population of granules or vesicles will have a range of fusion competencies. It may be inferred from this that granules/vesicles exposed to the same regulatory systems may irrespectively be subject to differential regulation. Further, it may be inferred that granules/vesicles exposed to differing regulatory systems may contribute to the same process.

Another important observation is that longer maturation does not necessarily increase the capacity of secretory granules to fuse with the plasma membrane. In fact it is likely more recently synthesised granules are released preferentially. Indeed, it has been reported that even immature granules are fusogenic (Tooze *et al.*, 1991). When a fluorescent 'timer' was expressed in adrenal chromaffin cells, targeted to secretory granules, granules of different ages could be distinguished through progressive changes in the emission spectra of this protein (Duncan *et al.*, 2003). When these cells were stimulated, it was reported that ~99% of the granules synthesised up to 2 hours previously were exocytosed compared to 0% of the granules older than ~16 hours, and ~69% of granules of an intermediate age (Duncan *et al.*, 2003). It may be inferred from this that granules do not 'queue' for fusion. Instead, it is likely that mechanisms promoting granule biosynthesis, trafficking, tethering and docking are temporally regulated, and accompanied by mechanisms promoting

continual turnover in the makeup of 'readily-releasable' and 'reserve' pools. Consequently, regulatory changes at any step in the secretory pathway may produce a more rapid effect. Further, if granules are subject to differential regulation according to their temporal, as well as their spatial localisation, the capacity of the system for precision is increased, and regulatory mechanisms may be combined and choreographed more easily.

1.4 Rab protein structure

Small GTPases of the Ras superfamily are present in eukaryotic but not prokaryotic cells (Leipe *et al.*, 2002). Because they are present throughout the eukaryotic lineage, it is thought that expansion and diversification within the superfamily occurred early in eukaryote evolution (Leipe *et al.*, 2002; Jekely, 2003). It has been found that different subfamilies within the superfamily can fulfil very different cellular functions, while the members of each subfamily may have distinct, but more related roles (Jekely, 2003). Major subfamilies include the Ras/Ral/Rap, Rho/Rac/Cdc42, Arf/Arl/Sar1, Rit/Rin, Ran, Rheb and Rab proteins. The Rab proteins (see table 1.1) comprise the largest of these subfamilies, and are largely found to function in membrane trafficking, while some may also function in signal transduction (Takai *et al.*, 2001; Schwartz *et al.*, 2007).

Despite differences in their cellular activities, the members of the Ras superfamily show a high degree of sequence homology, and are thought to

Isoform(s)	Localisation(s)	Function(s)	References
Rab1A-B	ER	ER to Golgi trafficking	(Tisdale *et al.*, 1992; Allan *et al.*, 2000)
Rab2A-B	ER	ER to Golgi trafficking	(Tisdale *et al.*, 1992)
Rab3A-D	Synaptic vesicles and secretory granules	Regulated exocytosis	(Schluter *et al.*, 2002; Tsuboi and Fukuda, 2006a; Rupnik *et al.*, 2007)
Rab4A-C	Early and recycling endosomes	Rapid recycling to plasma membrane	(van der Sluijs *et al.*, 1992; Bottger *et al.*, 1996; Mruk *et al.*, 2007)
Rab5A-C	Clathrin-coated vesicles, cavosomes and early endosomes	Endocytosis and early endosome fusion, caveolar vesicle targeting to early endosomes	(Bucci *et al.*, 1995; Barbieri *et al.*, 2000; Arnett *et al.*, 2004; Pelkmans *et al.*, 2004)
Rab6A	Golgi	Golgi-ER and intra-Golgi trafficking	(Martinez *et al.*, 1994; Jiang and Storrie, 2005; Del Nery *et al.*, 2006)
Rab6A'	Golgi	Endosome to Golgi trafficking	(Martinez *et al.*, 1994; Jiang and Storrie, 2005; Del Nery *et al.*, 2006)
Rab6B	Golgi	Golgi-ER and intra-Golgi trafficking	(Martinez *et al.*, 1994; Opdam *et al.*, 2000; Jiang and Storrie, 2005)
Rab6C	ERGIC-53 positive vesicles	multi-drug resistance regulation	(Martinez *et al.*, 1994; Shan *et al.*, 2000; Jiang and Storrie, 2005)
Rab7	Late endosomes, lysosomes	Trafficking to late endosomes from early endosomes; lysosome biogenesis	(Bucci *et al.*, 2000; Feng *et al.*, 2001)
Rab7B	Late endosomes, lysosomes	Traffic to lysosomes, TLR4 signaling	(Bucci *et al.*, 2000; Feng *et al.*, 2001; Wang *et al.*, 2007)
Rab8A-B	Post-Golgi vesicles; Golgi; endosomes; plasma membrane	Trafficking between Golgi and plasma membrane	(Peranen *et al.*, 1996; Chen *et al.*, 2001; Chen and Wandinger-Ness, 2001; Hattula *et al.*, 2006; Linder *et al.*, 2007; Nachury *et al.*, 2007)
Rab9A/B/C	Late endosomes	Trafficking to trans-Golgi; trafficking of lysosomal enzymes and cholesterol	(Lombardi *et al.*, 1993; Ganley *et al.*, 2004; Narita *et al.*, 2005)
Rab10	Golgi	Polarised membrane trafficking	(Chen *et al.*, 1993; Babbey *et al.*, 2006; Schuck *et al.*, 2007)
Rab11A	Golgi and recycling endosomes	Traffic from Golgi to apical recycling endosomes; phagocytosis	(Ullrich *et al.*, 1996; Wilcke *et al.*, 2000)
Rab11B	Golgi and recycling endosomes	calcium-dependent secretion	(Ullrich *et al.*, 1996; Wilcke *et al.*, 2000; Khvotchev *et al.*, 2003)
Rab12	Centrosomes	Trafficking from cell periphery to perinuclear centrosomes	(Iida *et al.*, 2005)
Rab13	Tight junctions; endosomes	Tight junction biogenesis	(Marzesco *et al.*, 2002)
Rab14	Rough ER; Gogi/trans-Golgi; early endosomes	Phagosome and early endosome fusion; trafficking between early endosomes and Glgi	(Junutula *et al.*, 2004; Kyei *et al.*, 2006; Proikas-Cezanne *et al.*, 2006)
Rab15	Early/sorting/recycling endosomes	Trafficking through recycling endosomes	(Zuk and Elferink, 2000; Elferink and Strick, 2005)
Rab17	Apical recycling endosomes in epithelia	Trandport through recycling endosomes; polarised sorting	(Zacchi *et al.*, 1998)
Rab18	Kidney dense apical tubules; Intestine basolateral membrane; ER lipid droplets; secretory granules	Formation of lipid droplets; negative regulator of secretion	(Lutcke *et al.*, 1994; Ozeki *et al.*, 2005; Vazquez-Martinez *et al.*, 2007)
Rab20	Kidney dense apical tubules	V-ATPase trafficking	(Curtis and Gluck, 2005)
Rab21	Early endosomes	Integrin endocytosis; cell-matrix interactions	(Pellinen *et al.*, 2006)
Rab22A	Early endosomes and trans-Golgi	Endosome to Golgi trafficking and phagosome transport	(Mesa *et al.*, 2001; Kauppi *et al.*, 2002; Roberts *et al.*, 2006)
Rab22B	Early endosomes and trans-Golgi	Trans-Golgi to endosome trafficking	(Mesa *et al.*, 2001; Rodriguez-Gabin *et al.*, 2001; Kauppi *et al.*, 2002; Roberts *et al.*, 2006)
Rab23	Plasma membrane, endosomes	embryogenesis; ciliaryy trafficking, sonic hedgehog trafficking	(Evans *et al.*, 2003)
Rab24	Autophagosomes, nuclear inclusions	Induction of autophagy	(Munafo and Colombo, 2002; Overmeyer and Maltese, 2005)
Rab25	Apical recycling endosomes in epithelia	Apical recycling endosome traffic	(Casanova *et al.*, 1999; Wang *et al.*, 2000)
Rab26	Secretory granules	Regulated exocytosis	(Yoshie *et al.*, 2000)
Rab27A-B	Secretory granules	Regulated exocytosis	(Barral *et al.*, 2002; Futter, 2006; Tsuboi and Fukuda, 2006a; Tolmachova *et al.*, 2007)
Rab32	Perinucluar vesicles, mitochondria	Post-Golgi traffic; mitochondrial dynamics	(Alto *et al.*, 2002; Wasmeier *et al.*, 2006)
Rab33A-B	Medial Golgi	Golgi-ER and trafficking	(Valsdottir *et al.*, 2001)
Rab34	Membrane ruffles; lysosomes	Macropinosome formation and regulation of lysosome location	(Sun *et al.*, 2003; Colucci *et al.*, 2005; Sun and Endo, 2005; Wu *et al.*, 2005)
Rab37	Secretory granules	Mast cell degranulation	(Masuda *et al.*, 2000; Brunner *et al.*, 2007)
Rab38	Tyrosinase-positive vesicles	Melanosome biogenesis	(Wasmeier *et al.*, 2006)

Table 1.1. Rab protein functions. Modified from Shwartz *et al.*, 2007.

be structurally similar (Stenmark and Olkkonen, 2001). Each protein is thought to consist of a six-stranded β-sheet containing five parallel strands and one antiparallel strand, and surrounded by five α-helices (Pai *et al.*, 1989; Dumas *et al.*, 1999; Stenmark and Olkkonen, 2001; Pfeffer, 2005c). Loops connecting the α-helices and β-strands contain elements responsible for GTP/GDP binding, GTPase activity and Mg^{2+}-binding, and the 'active site' of each protein contains conserved residues that interact with nucleotide phosphate groups, Mg^{2+}, and the guanine base (Pai *et al.*, 1989; Dumas *et al.*, 1999; Stenmark and Olkkonen, 2001). Regions denoted 'Switch I' and 'Switch II' are found to surround the γ-phosphate of GTP where this nucleotide is bound, while GTP-hydrolysis produces dramatic conformational change (Milburn *et al.*, 1990; Stroupe and Brunger, 2000).

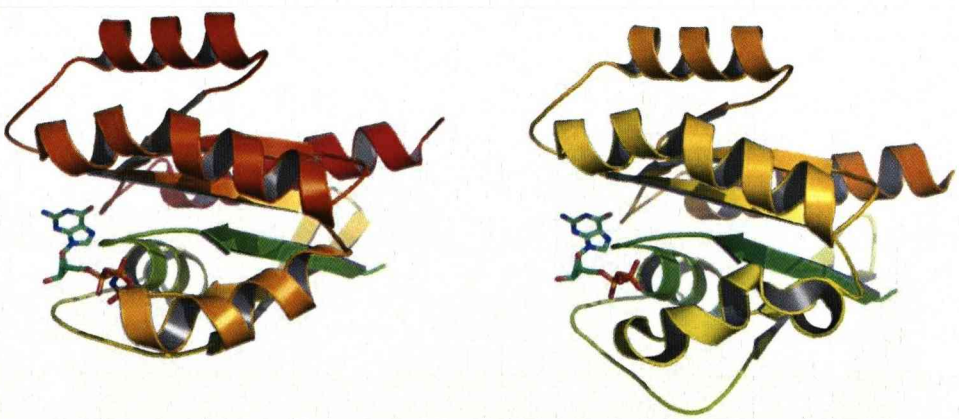


Figure 1.7. Crystal structures of Rab protein Sec4 bound to GTP-analogue GppNHp (left) and GDP (right). Structures demonstrate dramatic conformational differences. Molecular coordinates taken from Stroupe and Brunger, 2000 (RCSB PDB Structure IDs 1G16 and 1G17)

The divergent functions of small GTPase subfamilies are often reflected by divergent subcellular localisations. These localisations may be partly dependent on differential post-translational modification. For example, Arf

proteins are myristoylated at an amphipathic N-terminal helix (Takai *et al.*, 2001). This myristoylated domain mediates membrane association, while conformational changes induced by GTP-binding enhance this association (Kahn *et al.*, 1991; Takai *et al.*, 2001). Nascent Rab proteins undergo geranylgeranylation at two Cys residues within a C-terminal cysteine-rich hypervariable region, and the terminal Cys of this region may undergo carboxymethylation (Takai *et al.*, 2001; Goody *et al.*, 2005). A Rab escort protein (REP) is required to present each Rab protein to the geranylgeranyl transferase (GGTase) enzyme for modification, while the targeting of the modified protein to specific membranes may require additional factors (see below). It was originally thought that the hypervariable region conferred additional specificity to Rab-membrane targeting (Chavrier *et al.*, 1991). More recently, it has been reported that multiple regions contribute to this specificity, while it has been suggested that the hypervariable regions are relatively unstructured and may have a 'tethering' function (Ali *et al.*, 2004; Pfeffer, 2005b). That is, their prenylation may lead to Rab membrane-association, while they may also provide a flexible 'link' allowing the structured portion of the Rab protein to operate at some distance from the membrane (Pfeffer, 2005b).

1.5 Rab protein function

The first work showing the importance of Rab proteins in membrane trafficking came from studies in Saccharomyces cerevisiae (yeast). It was

shown that mutations in Sec4 could cause accumulation of post-Golgi vesicles and a block in their delivery to the plasma membrane, while mutations in the homologous Ypt1 could produce a similar block in traffic from the ER to the Golgi (Salminen and Novick, 1987; Segev *et al.*, 1988). It has since been shown that deletion of genes encoding any of the yeast Rab proteins functioning in the biosynthetic pathway is lethal (Lazar *et al.*, 1997). In mammals, deletion of a particular Rab isoform can produce variable and less dramatic effects, with deletion of Rab3A in the mouse, for example, producing no effects on viability, and only minimal effects on neurotransmitter release (Geppert *et al.*, 1994a). It is possible that this may reflect increasing overlap or specialisation in the roles of specific isoforms, and that this corresponds to increasing complexity in membrane trafficking in these organisms (Zerial and McBride, 2001; Gurkan *et al.*, 2005). In the course of mammalian evolution, there has been a dramatic expansion in the Rab protein family, with over 60 Rabs identified in mammals compared to only 11 in yeast (Grosshans *et al.*, 2006). This may indicate that some mammalian Rab proteins may sometimes serve redundant and/or non-essential cellular roles.

Expression profiling has been used to correlate the expression of specific Rab isoforms, and that of other proteins, also linked with the function of particular membrane compartments or with the mediation of particular steps in membrane trafficking (Gurkan *et al.*, 2005). Further, the association of a specific Rab(s) with particular membrane compartments/trafficking steps is consistent with their localisation in cells,

which is usually correspondingly restricted (Zerial and McBride, 2001; Ali *et al.*, 2004). They are found to direct varied processes through a wide range of effector proteins, and are usually involved in the recruitment of these effectors to membranes.

The functional specialisation of different membrane compartments can be maintained by their continual maturation, and/or through the continual trafficking of transport vesicles between them (Pfeffer, 2003). A key role of Rabs and their effectors is thought to be in conferring specificity on these processes. For example, a particular vesicle-associated Rab protein may bind to effectors regulating multiple events in vesicle targeting to a specific target compartment conferring 'compartmental specificity' on vesicle trafficking (Grosshans *et al.*, 2006). Other factors with a restricted compartmental localisation, such as the SNARE proteins, may also mediate 'compartmental specificity' (McNew *et al.*, 2000). However, the cyclical manner by which Rab proteins are recruited and activated on membranes may be critical for establishment and maintenance of the necessary asymmetry in trafficking. Where other proteins require trafficking to their target compartments, they may be less likely to adopt these roles (Munro, 2002).

1.6 The Rab GTPase cycle

As described above, small GTPases adopt distinct conformations according to whether they are GDP-, or GTP-bound (Milburn *et al.*, 1990). As distinct GTPase conformations can participate in distinct protein-protein interactions, this characteristic allows these proteins to function as molecular 'switches' or 'timers'. That is, regulation of their nucleotide-bound states can reversibly limit or alter specific cellular activities. For this reason, small GTPases often act in signalling cascades. For example, mitogenic signalling can lead to association between Ras and GTP, and GTP-Ras, through recruitment of a kinase, Raf, can promote phosphorylation within the MAPK/ERK pathway (Stokoe *et al.*, 1994; Takai *et al.*, 2001).

Ras GTP-association is important in promoting many downstream processes including proliferation and migration (Takai *et al.*, 2001). Mutations that directly or indirectly favour inappropriate Ras GTP-association are often linked to excessive proliferation and migration, and often found in cancers, indicating that cyclical GDP/GTP association may be dispensable for the function of Ras in signal transduction and amplification (Takai *et al.*, 2001). However, cyclical GDP/GTP association are important for the functions of other small GTPases. For example, nuclear GTP-Ran is involved in the export of cargo molecules from the nucleus, while cytosolic Ran GTP-hydrolysis is required for its dissociation from the cargo and import back into the nucleus. In this case, mutations

favouring Ran GTP-association inhibit the nuclear export process (Schlenstedt *et al.*, 1995; Carey *et al.*, 1996).

Like Ras, it is found that Rab proteins typically interact with their effector proteins when in a membrane-associated, GTP-bound state. However, it is most likely that they are efficiently targeted to specific membrane compartments in a GDP-bound state. Rab protein targeting, nucleotide exchange and GTP hydrolysis are presented in Fig.1.8, and discussed in more detail below.

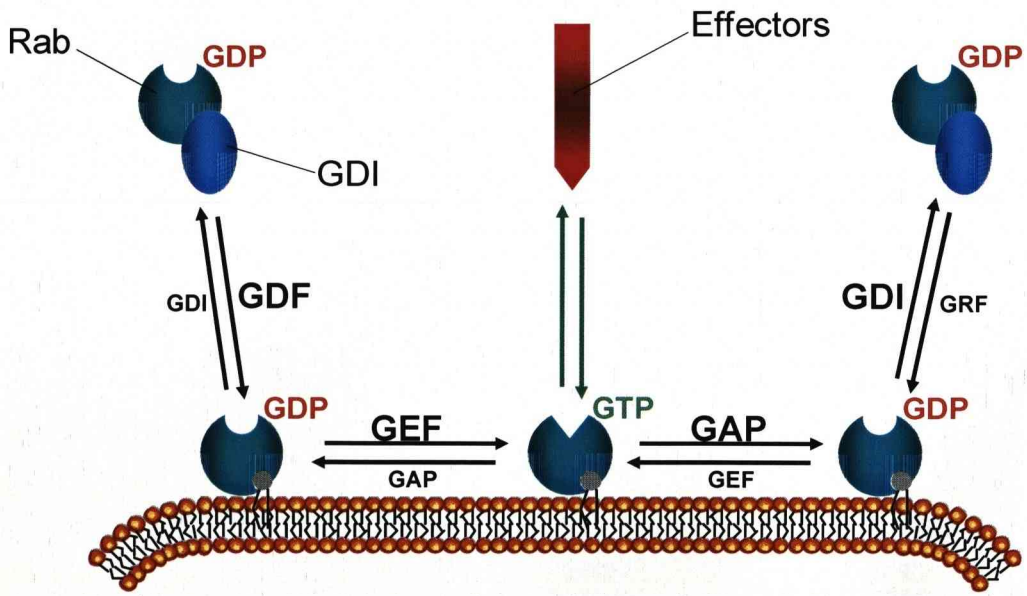


Figure 1.8. Conventional model for Rab protein function. Within the cytosol, Rab proteins are sequestered in a GDP-bound state by GDP dissociation inhibitor proteins (GDIs) which are involved in their extraction from membranes and also in the targeting of specific Rab proteins to specific membrane compartments through a mechanism also thought to require a GDI dissociation factor (GDF). Once membrane associated, GDP-bound Rab proteins may become GTP-bound through the action of a GDP to GTP exchange factor (GEF), and it is typically the GTP-bound Rab protein that interacts with effector proteins. A GTP-bound Rab protein may become GDP-bound though interaction with a GTPase-activating protein (GAP) that stimulates its intrinsic GTPase activity.

1.6.1 Targeting

Rab proteins can show highly specific subcellular localisation to particular membrane compartments (Ali *et al.*, 2004). It is found that their isoform-specific targeting can depend on distinct domains, while it is also found that alone, prenylated Rab proteins can associate with isolated cellular membranes indiscriminately, demonstrating a requirement for an additional cellular factor(s) in the targeting process (Dirac-Svejstrup *et al.*, 1994; Ali *et al.*, 2004). In a GDP-bound state, Rabs and some other small GTPases may be sequestered in the cytosol by GDP dissociation-inhibitor (GDI) proteins (Ullrich *et al.*, 1993; Takai *et al.*, 2001; Goody *et al.*, 2005). While limiting their activity, these proteins can be involved in the appropriate targeting of GTPases to specific subcellular localisations. For example, RhoGDI may be displaced from Rho proteins by the ezrin/radixin/moesin (ERM) complex, and its subsequent activation can lead to ERM translocation to the plasma membrane (Takahashi *et al.*, 1997).

RabGDIs have a structure unlike that of RhoGDIs, but similar to REP proteins (Schalk *et al.*, 1996; Keep *et al.*, 1997; Goody *et al.*, 2005). They are able to form soluble complexes with GDP-bound Rab proteins through sequestration of their hydrophobic prenyl groups, and so may prevent their inappropriate membrane-association, and mediate their membrane-extraction (Schalk *et al.*, 1996; Goody *et al.*, 2005). They may also be indirectly or directly involved in selective targeting of Rab proteins, because Rab5- and Rab9-GDI complexes are correctly targeted in vitro (Dirac-Svejstrup *et al.*, 1994; Soldati *et al.*, 1994; Ullrich *et al.*, 1994).

However, only three Rab-GDI isoforms are identified in mammals, GDIα, GDIβ and GDIγ, and it has been suggested that these proteins act as general regulators of Rab protein function (Ullrich *et al.*, 1993). Therefore, the specific targeting of Rab proteins is likely to involve their isoform-specific interactions with other factors.

It has been suggested that some additional selectivity in Rab targeting may be conferred by transiently-acting membrane-associated GDI-displacement factors (GDFs), which are required to mediate Rab-membrane association (Pfeffer and Aivazian, 2004). In yeast, Yip1p may act as a GDF for a subset of Rab proteins targeted to the Golgi (Chen *et al.*, 2004). In mammals, PRA1/Yip3 is shown to displace GDI from Rab proteins of the endosomal pathway including Rab9 and Rab5, and is required for their recruitment to membranes (Dirac-Svejstrup *et al.*, 1997; Sivars *et al.*, 2003). However, since these Rabs are targeted to distinct endosomal compartments it is unclear how their localisation is further restricted (Pfeffer and Aivazian, 2004; Behnia and Munro, 2005). One possibility is that Rab proteins are trafficked to the appropriate compartment after associating with membranes in a specific trafficking pathway (Pfeffer and Aivazian, 2004). Alternatively, it has been suggested that the sustained association of Rab proteins with their target compartment might be dependent on nucleotide exchange (Behnia and Munro, 2005).

1.6.2 Nucleotide exchange

Following GDI displacement, GDP-bound Rab proteins associated with membranes may be subject to GDP→GTP exchange catalysed by guanine nucleotide exchange factors (GEFs)(Takai *et al.*, 2001). When in a GDP-bound state, membrane-associated Rabs may be susceptible to GDI-mediated membrane extraction, but they do not interact with GDIs when GTP-bound. Any association of an isoform-specific RabGEF and a particular membrane compartment might therefore serve to selectively concentrate a particular Rab protein on this compartment, if its GDI-dependent extraction from other compartments is rapid. Further, Rab protein effectors, through which Rabs mediate their cellular functions, almost always interact with only the GTP-bound form of a given Rab protein, and so nucleotide exchange may couple selective targeting of Rab isoforms with their selective 'activation' (Carney *et al.*, 2006).

GEFs for all of the Rab protein isoforms present in yeast have been identified, while the GEFs acting on specific mammalian Rab isoforms are largely unknown (Carney *et al.*, 2006). The RabGEFs identified in yeast are found to share no sequence homology, while they are often found to function as components of large multi-subunit complexes (Jones *et al.*, 2000; Carney *et al.*, 2006). Each GEF usually has Rab isoform-specific GEF activity, although the transport protein particle (TRAPP) complex is reported to act on Ypt1, Ypt31 and Ypt32 (Jones *et al.*, 2000). The seven-subunit TRAPPI complex is thought to act preferentially on Ypt1, and to be involved in regulating the entry of cargo vesicles into the Golgi (Morozova

et al., 2006). The maturation of the TRAPPI to the TRAPPII complex, through its association with three additional subunits, changes its specificity and is required for its regulation of Ypt31/32, and cargo exit from the Golgi (Morozova *et al.*, 2006). Thus, the TRAPP complex appears to link sequential steps in membrane trafficking.

Several other RabGEFs also appear to link sequential steps in membrane trafficking, though they do so by linking sequential Rab protein functions more directly. In yeast, the Ypt31/32 effector Sec2 has been identified as a GEF for the Rab protein Sec4, which functions in the transport and fusion of post-Golgi vesicles with the plasma membrane (Walch-Solimena *et al.*, 1997; Ortiz *et al.*, 2002). Similarly, mammalian Rab5 is found to interact with one subunit of the class C VPS/HOPS complex, another subunit of which acts as a GEF for Rab7 (Rink *et al.*, 2005). In the transition from Rab5-positive early endosomes to Rab7-positive late endosomes, it is thought that Rab5 mediates the membrane-recruitment of Rab7 through the activity of this complex, and is subsequently replaced by it (Rink *et al.*, 2005).

If the systems described above represent typical examples, it might be suggested that Rab protein recruitment and/or activation initiates and directs discrete membrane trafficking processes. Consistent with this suggestion, it was found that the delivery of vesicles into the same trafficking pathway can be modulated through activation of a particular Rab isoform by multiple RabGEFs, according to cellular requirements (Carney

et al., 2006). For example, several proteins with GEF activity on Rab5 have been characterised, and have distinct roles. It is likely that Rabex5 is general regulator of Rab5-directed fusion of endocytic vesicles with early endosomes, and homotypic fusion between early endosomes (Carney *et al.*, 2006). RME-6 localises to Clathrin-coated pits and may be required earlier in the endocytotic pathway (Sato *et al.*, 2005). In contrast, Rin1 more selectively binds to EGFRs and H-Ras, and its Rab5GEF activity, and thereby Rab5-directed EGFR internalisation, is potentiated where Ras is active (Tall *et al.*, 2001). Alsin and ALS2CL are thought to be involved in selective Rab5-directed endocytosis of receptors in the nervous system, while Alsin may coordinate nucleotide exchange at Rab5 and Rac (Hadano *et al.*, 2004; Topp *et al.*, 2004).

1.6.3 GTP hydrolysis

Rab proteins possess constitutive GTPase activity, and so may in principle hydrolyse bound GTP to GDP autonomously (Takai *et al.*, 2001). In fact, however, their GTP hydrolysis is usually low and is regulated physiologically by GTPase activating proteins, GAPs, which accelerate the reaction (Takai *et al.*, 2001). Unlike RabGEFs, RabGAPs with differing Rab isoform-specificity typically share conserved Tre-2, Bub-2, Cdc16 (TBC) domains which are responsible for their activity, and this has allowed the identification of many putative mammalian RabGAPs through sequence and functional analysis (Haas *et al.*, 2005; Itoh *et al.*, 2006). However, the in vivo specificity of the majority of these proteins has not yet been fully characterised.

Rab GTP-hydrolysis is classically associated with the termination of discrete membrane trafficking steps, since reduction in the levels of a GTP-bound Rab protein on a given membrane attenuates the activity of its effectors, while GDP-bound Rab protein may be extracted from membranes in a GDI-dependent manner (Goody *et al.*, 2005). In order to regulate this process, a specific RabGAP may be localised to the last membrane compartment to which a given Rab isoform directs traffic, or the Rab protein may be relatively shielded from GAP activity prior to reaching this compartment. It has often been suggested that the association between GTP-bound Rab proteins and their effectors might generally shield them in this manner (Zerial and McBride, 2001; Grosshans *et al.*, 2006).

The continued characterisation of Rab protein function has shown that some Rab proteins can control multiple sequential events in a particular trafficking step through interaction with distinct effectors or groups of effectors (Zerial and McBride, 2001; Schwartz *et al.*, 2007). In these cases, it may be that RabGAP activity and GTP-hydrolysis are associated with completion of each event. For example, GAP activity might underlie the observation that when levels of a GFP-tagged Rab5 fusion protein were tracked on single early endosomes, increases in individual endosomal levels of this protein that resulted from endosome-endosome fusion often declined rapidly following the fusion event, in the apparent absence of associated fission events (Rink *et al.*, 2005). Since fusion between

endosomes is controlled by Rab5, this suggestion is consistent with findings showing that depletion of RabGAP-5, or expression of mutated Rab5 deficient in GTPase activity, leads to endosome enlargement and accumulation (Stenmark *et al.*, 1994; Roberts *et al.*, 1999; Haas *et al.*, 2005). It is found that many rounds of endosome-endosome fusion may occur prior to the eventual loss of Rab5 when early endosomes mature into late endosomes (Rink *et al.*, 2005). Thus, it is likely that GEFs and GAPs function in concert to dynamically maintain Rab5 levels appropriate for early endosome function, rather than functioning separately to promote its association with early endosomes, and its exclusion from late endosomes, respectively.

Convergence of opposed GEF and GAP activity on the same membrane compartment implies the possibility of a further level of complexity in the regulation of Rab protein nucleotide-binding and membrane-association. Upon association with their target membranes, GDP-bound Rab proteins may be subject to either GEF-mediated nucleotide exchange or GDI-mediated membrane-extraction. GTP-bound Rab proteins may hydrolyse GTP through interaction with a GAP, or may be stabilised to a variable degree through interactions with different effectors. Thus, 'basal' Rab-membrane association and dissociation may occur. In these circumstances, GEF and GAP activities would be determinants of 'basal' Rab association and dissociation rates, in addition to controlling the net extent of Rab dissociation following specific Rab-controlled events.

The dynamics of Rab proteins functioning in secretory granule exocytosis is a major subject in this thesis, and will be discussed later with reference to experimental data presented here. However, there is support for dynamic Rab-membrane association and dissociation under 'resting' conditions from a number of studies focusing on other Rab isoforms. In a relatively early study, it was found that a GTP-analogue bound to Rab5 underwent hydrolysis even in the absence of endosome-endosome fusion (Rybin *et al.*, 1996). This suggests that a fraction of endosomal Rab5 may continually become susceptible to GDI-mediated extraction, and indeed, overexpression of RabGAP-5 or GDI can 'strip' Rab5 from endosomal membranes (Ullrich *et al.*, 1993; Ullrich *et al.*, 1994; Haas *et al.*, 2005).

More recently, a model has been proposed by which the regulation of RabGAP activity, and Rab dynamics, might control GLUT4 translocation in adipocytes. It is suggested that the RabGAP TBC1D4, and possibly also TBC1D1, limit the GTP-binding and/or membrane-association of Rab(s) targeted to GLUT4 carrier vesicles (Kane *et al.*, 2002; Roach *et al.*, 2007). TBC1D4 is found to be associated with, and essential for the intracellular retention of these vesicles in unstimulated cells, while stimulation of cells with insulin may lead to activation of the protein kinase Akt, and TBC1D4 phosphorylation (Sano *et al.*, 2003; Eguez *et al.*, 2005; Larance *et al.*, 2005). Akt phosphorylation of TBC1D4 reduces its GAP activity in a 14-3-3-dependent manner, and it is suggested that this could lead to increased Rab10 recruitment or activation on these vesicles, initiating vesicle delivery to the plasma membrane (Ramm *et al.*, 2006; Sano *et al.*, 2007).

1.7 Effector interactions and Rab domains

Rab proteins can function through various effectors in the initiation and progression of discrete steps in membrane traffic. It is found through sequence analysis of the Rab family that there is a high degree of conservation in amino acid residues likely to surround the switch regions, while surface residues peripheral to this area show more divergence (Pereira-Leal and Seabra, 2000). On the basis of this analysis, it has been suggested that while effectors might interact with Rab proteins at the switch regions, and typically interact stably only where GTP is bound, the binding specificity of a given effector for a particular Rab isoform(s), may be conferred by a distinct protein-protein interaction (Pereira-Leal and Seabra, 2000). Consistent with this suggestion, published crystal structures of Rab proteins interacting with specific effectors show alternate modes of interaction (Ostermeier and Brunger, 1999; Zhu *et al.*, 2004; Jagoe *et al.*, 2006). Further, comparison of identified Rab effectors shows that those binding to divergent Rab proteins may have very different structures. Indeed, even effectors binding to the same Rab isoform do not necessarily show any sequence similarity (Gonzalez and Scheller, 1999; Pereira-Leal and Seabra, 2000). However, it is shown that some effector families can share homologous sequence motifs essential for their binding to a particular Rab protein isoform, or several closely related isoforms (Izumi *et al.*, 2003; Fukuda, 2005).

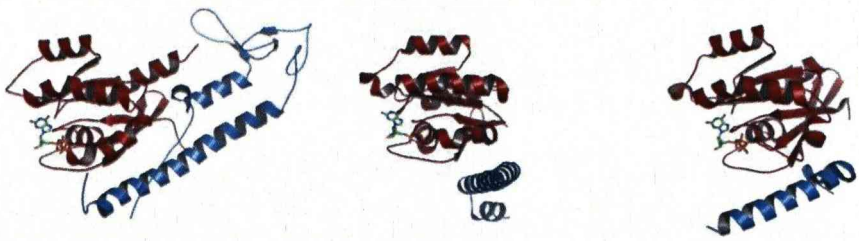


Figure 1.9. Structural differences define the specificity in binding of Rabs to effectors, while nucleotide-binding regions show greater conservation. Crystal structures of Rab3A in a complex with a fragment of Rabphilin (left), Rab5 in a complex with a fragment of Ranaptin5 (centre), and Rab11 in a complex with a fragment of FIP2 (right). Molecular coordinates were taken from Ostermeier and Brunger, 1999; Zhu et al., 2004 and Jagoe et al., 2006. (RCSB PDB Structure IDs 1ZDB, 1TU3 and 2GZD)

It is usually found that effectors are recruited from the cytosol by activated Rabs, and that their Rab binding specificity is important in their recruitment to appropriate membrane compartment(s). Typically, the in vitro binding specificity of a Rab effector mirrors that identified in vivo, and it is also found that the deletion of a particular Rab isoform can affect effector stability (Kuroda *et al.*, 2002; Fukuda, 2003a). Where Rab3A is absent from neurones, for example, levels of its effector Rabphilin are reduced by ~50% (Li *et al.*, 1994). Conversely, effector deletion can sometimes affect Rab stability. For example, there was a 4-fold decrease in Rab9 half-life where TIP47 levels were reduced with siRNA, and this may support the suggestion that Rabs and effectors promote reciprocal target-interactions (Ganley *et al.*, 2004; Aivazian *et al.*, 2006). This would be consistent with the prior suggestion that Rab-effector interactions, as well as the activities of specific GEF and GAP proteins, might be determinants of the activity and membrane-association of particular Rab isoforms.

Effector-target interactions may also be contingent on other factors. TIP47 binds to membrane-associated mannose-6-phosphate receptors (MPRs), for example, in order to direct cargo selection, and its affinity for these receptors is higher when it is simultaneously bound to Rab9 (Carroll *et al.*, 2001). Rab9 and TIP47 may therefore be preferentially recruited to MPR-containing membrane subcompartments, the site of their combined function. Furthermore, this kind of 'multi component requirement' for recruitment may serve to restrict the activity of effectors to a greater extent than that of their target Rab. Thus, it may be one mechanism by which the activity of effectors involved in separated or sequential events coordinated by the same Rab isoform is regulated.

It has been shown that the localisation of membrane-associated Rab9 and TIP47 is not only restricted to a particular membrane subcompartment, but also to tightly packed 'Rab domains' on the surface of membranes (Barbero *et al.*, 2002). In the case of these proteins, this characteristic may relate to their role in concentrating proteins in cargo selection at the late endosome, while many other Rabs are also characterised as components of such 'domains'. On early endosomes and on recycling endosomes, Rabs 4 5 and 11 form distinct subdomains, on early/sorting endosomes, Rabs 4 and 5 form distinct domains, and on late endosomes, Rabs 9 and 7 form distinct domains, for example (Sonnichsen *et al.*, 2000; Barbero *et al.*, 2002). Perhaps the best characterised Rab protein known to form 'Rab domains' is Rab5 (Zerial and McBride, 2001; Grosshans *et al.*, 2006).

Rab5 is recruited to clathrin-coated endocytotic vesicles and early endosomes, and is involved in tethering and fusion of endocytotic vesicles and early endosomes, and homotypic fusion between endosomes (Ullrich *et al.*, 1994; Horiuchi *et al.*, 1995; Christoforidis *et al.*, 1999; Nielsen *et al.*, 2000). It is thought that Rab5 'domain' formation is initiated as the result of a signalling cascade downstream of its binding to GTP. GTP-Rab5 can recruit the effector protein Rabaptin-5, which can in turn recruit the Rab5 GEF Rabex5, thereby locally stabilising GTP-Rab5 through positive feedback (Stenmark *et al.*, 1995; Lippe *et al.*, 2001; Grosshans *et al.*, 2006). The positive effect of Rabaptin-5 on Rab5 activation is supported by the finding that its overexpression leads to enlargement of endosomes; the same phenotype as seen where a consitutively active Rab5 is expressed, or where RabGAP-5 is subjected to siRNA knockdown (Stenmark *et al.*, 1994; Stenmark *et al.*, 1995; Haas *et al.*, 2005). It has been found that constitutively active Rab5 domains localise to the interface between fusing endosomes, and these domains can persist as a localised spot following endosome-endosome fusion (Roberts *et al.*, 1999).

On early endosomes, GTP-Rab5 may recruit p150 phosphatidylinositol (PI) 3-OH kinase/hVps34 and/or PI3K, leading to generation of PI(3)P on endosomal membranes (Simonsen *et al.*, 1998; Zerial and McBride, 2001). Since PI(3)P is required for the stable membrane association of Rab5 effectors EEA1 and Rabenosyn5, and is not present on Clathrin-coated endocytotic vesicles, their stable recruitment by GTP-Rab5 is limited to early endosomes (Nielsen *et al.*, 2000). The fusion of endocytotic vesicles

with early endosomes is dependent on EEA1, and this effector can substitute for cytosol in early endosome fusion assays (Christoforidis *et al.*, 1999; Rubino *et al.*, 2000). EEA1 is a tethering protein, and forms a homodimer on which two binding sites each for PI(3)P and Rab5 are located at one end of a large coiled-coil region (Callaghan *et al.*, 1999). As a component of 'Rab5 domains', it has been suggested to associate with the SNARE protein Syntaxin13, and transiently with NSF, and these interactions are thought to be essential for early endosomal fusion (McBride *et al.*, 1999). It has been suggested that while stable EEA1-containing domains on early endosomes might promote homotypic fusion, distinct Rab5 domains on Clathrin-coated endocytotic vesicles may represent low affinity EEA1 binding sites, and this may confer specificity on their tethering to these organelles (Rubino *et al.*, 2000).

In a number of cases, as noted above, distinct Rab isoforms may form 'domains' at the same membrane compartment. Thus, in addition to serving as organisational platforms for effectors coordinating specific events, Rab domains may also serve to separate the independent functions of distinct Rab effectors. However, several Rab effectors have been shown to interact with multiple Rab isoforms. For example, Rabaptin5 and Rabenosyn each bind both rab4 and rab5, while RCP binds both Rab4 and Rab11 (Vitale *et al.*, 1998; de Renzis *et al.*, 2002; Lindsay *et al.*, 2002). It has been suggested that such effectors link different Rab domains, and may in this way function in the retention of multiple Rabs on one membrane (de Renzis *et al.*, 2002; Pfeffer, 2003). Different Rab

isoforms on a given membrane compartment may appear to have independent functions, functioning in the entry and exit of distinct transport vesicles, for example. However, in this example, their role in maintenance of the composition and function of the compartment is interdependent, since loss of either Rab would lead to formation of a terminal compartment (Pfeffer, 2003).

1.8 Summary

It is evident from the above discussion that considerable progress has been made in the characterisation of the function and regulation of specific Rab proteins. Nevertheless, there is still a significant amount to be learnt about the dynamics and effector interactions of each Rab protein isoform, and each of their functional roles in membrane trafficking. In general, Rab proteins appear to confer directionality in intra- and intercompartmental membrane trafficking, and to act as versatile platforms for the organisation of compartment-specific sorting, trafficking and fusion machinery. From the earlier discussion of regulated exocytosis, it is clear that diverse events can exquisitely control the biogenesis of secretory granules and synaptic vesicles, their approach to the plasma membrane, their fusion potential, and multiple aspects of the fusion process itself. While several Rab protein isoforms are suggested to function in exocytosis, and a number of their effectors have been identified and functionally characterised, a fully integrated picture of Rab protein function in the process is yet to emerge.

1.9 Aims and objectives

The broad aim of this study was to explore the function, regulation and dynamics of Rab3A, Rab27A, and a number of their effectors. More specific aims were as follows:

1. To characterise the localisation of fluorescently-tagged Rab3A and Rab27A, their recruitment onto secretory granules, their effects on exocytosis, and their dynamics in resting and stimulated cells.

2. To characterise the localisation and fluorescently-tagged versions of the Rab effectors Rabphilin, Granuphilin, Slp5 and Noc2, and to analyse their dynamics in live cells. To characterise the functional effects of a novel Rab3A interactor Munc18-1.

3. To characterise proteins reported to act as GAPs for Rab3A and Rab27A, and to screen multiple TBC domain-containing proteins in order to identify additional candidate GAPs.

Chapter 2:

Materials and Methods

2.1 Molecular Biology

2.1.1 cDNA preparation

Total PC12 cell RNA was prepared from ~10^6 PC12 cells. Cells were sedimented by cetrifugation at 800 x g for 5 minutes, and then resuspended and lysed in 1ml TRIzol Reagent (Invitrogen, Paisley, UK). Following incubation at room temperature for 5 minutes, 200μl of chloroform was added, and the mixture was incubated for a further 2 minutes. The mixture was then centrifuged at 12000 x g for 15 minutes at 4°C, causing it to separate into organic and aqueous phases. The RNA-containing aqueous phase was removed to 500μl isopropanol to precipitate the RNA. Following incubation at room temperature for 10 minutes, RNA was sedimented by centrifugation at 12000 x g for 10 minutes, and the pellet was washed with 1ml 75% EtOH. The purified RNA was sedimented by centrifugation at 7500 x g for 5 minutes, then partially air-dryed and finally dissolved in deionised, RNAse-free water.

cDNA was prepared using 1μg of the above RNA preparation in each reaction. The RNA, and 200pmol oligo dT(15) primers (Promega, Southampton, UK) were mixed in 5μl distilled water, on ice, and the template was prepared by incubating the mixture at 70°C for 5 minutes, and then rapidly cooling on ice. 15μl of a buffer solution was then added to give a reaction mixture containing 4μl ImPromII 5x buffer (Promega), 3mM $MgCl_2$, 0.5mM dNTPs and 1μl (1 reaction) ImProm-II Reverse Transcriptase (Promega). This reaction mixture was incubated at room

temperature for 5 minutes and then at 42°C for 1 hour. The resulting cDNA preparation was then incubated at 70°C for 15 minutes in order to inactivate the reverse transcriptase, and then used as a template for PCR.

2.1.2 PCR

For cloning from cDNA prepared as above, or subcloning from existing plasmids, KOD polymerase (Novagen, Nottingham, UK) was used in the PCR-mediated amplification of specific sequences. Each 50μl reaction mixture contained 5μl 10x PCR buffer (Novagen), 0.2mM dNTPs, 1mM $MgSO_4$, 0.3μM forward and reverse primers and 1U KOD polymerase in distilled water. 1μl PC12 cell cDNA, or 50ng plasmid DNA were used as template. The PCR conditions used were as follows:

1 cycle:	98°C	2 minutes
35 cycles:	98°C	15 seconds
	55°C	30 seconds
	68°C	20 seconds per kb product
1 cycle	72°C	5 minutes
	30°C	1 minute

2.1.3 Restriction Endonuclease Digestion

For digestion of PCR products and vectors prior to ligation, or for restriction analysis of recombinant plasmids, the following conditions were used. Digestion mixtures containing 4μl of the appropriate 10x buffer (Promega, Southampton, UK), 1μl (10U) of each enzyme used (Promega), and up to

1μg DNA, in 40μl distilled water, were made up on ice and then incubated at 37°C for 2 hours. Products of digestion were separated by agarose gel electrophoresis, and purified by gel extraction where required.

2.1.4 Agarose gel electrophoresis and gel extraction

For separation of DNA fragments, for purification or analysis, agarose gel electrophoresis was used. Gels were made with 1% (v/w) agarose in TAE buffer (40mM Tris base pH 8, 20mM acetic acid, 1mM EDTA). To label DNA, gels contained 0.5μg/ml ethidium bromide. DNA samples of up to 50μl were combined with 2-10μl sucrose prior to running, and run against the Hyperladder I molecular weight marker (Bioline, Sheffield, UK) at 70-120V. For purification of DNA fragments from gels, QIAquick gel extraction kits (Qiagen, Crawley, UK) were used. 'Bands' cut from gels were initially dissolved to release DNAs, then the DNAs were bound to an anion exchange column, washed, and eluted in 30μl distilled water.

2.1.5 Ligation

Ligation reactions were used to generate circular DNAs from linear DNA fragment(s), typically for cloning or subcloning through insertion of a coding insert into a given vector. Reactions were carried out using 100ng vector DNA, with a 1:3 molar ratio of vector:insert DNA. The reactions were carried out by addition of a mixture of 5μl 2x Rapid Ligation buffer (Promega, Southampton, UK), 1μl (1-3U) T4 DNA Ligase (Promega) and distilled water to a final volume of 10μl, and incubation at room

temperature for 30-60 minutes. The entire reaction mixture was then used to transform chemically competent *E.coli.*

2.1.6 Transformation of chemically competent *E. coli*

Where the products of a ligation reaction were used for transformation, XL-1 blue cells (Stratagene, Cambridge, UK) were used. For each transformation, 100μl of cells were thawed on ice and then added to 1.7μl of the provided β-mercaptoethanol (Stratagene). Following 10-15 minutes incubation on ice, ligation products were added, and the suspension was incubated on ice for a further 30 minutes. Cells were then subjected to a 42°C heat-shock for 45 minutes, retuned to ice briefly, and then added to 900μl prewarmed SOC medium (20g/l tryptone, 5g/l yeast extract and 500mg/l NaCl in distilled water). This suspension was then incubated at 37°C for 1 hour, with shaking at 250RPM. Various volumes were then spread on LB-Agar plates (20g/l Agar, 10g/l tryptone, 10g/l NaCl and 5g/l yeast extract in distilled water) containing an appropriate antibiotic for selection (100μg/ml ampicillin or 25μg/ml kanamycin). Transformant clones were picked following overnight incubation at 37°C.

For routine transformation using plasmid DNA, TurboCells (Gene Therapy Systems Inc., San Diego, USA) were used. For each transformation, 50μl of cells were thawed on ice, and ~50ng vector DNA added. Following heat-shock at 37°C for 1 minute, 200μl TurboCells transformation buffer (Gene Therapy Systems) was added, and cells were spread on LB-Agar plates as above.

2.1.7 Purification of plasmid DNA

In order to produce small plasmid preparations from transformant clones (see above), single clones were used to inoculate 4ml LB medium (10g/l tryptone, 10g/l NaCl and 5g/l yeast extract in distilled water) containing an appropriate antibiotic (100μg/ml ampicillin or 25μg/ml kanamycin), and these cultures were then grown overnight at 37°C with shaking at 250RPM. 3ml of each stationary culture was sedimented by centrifugation (12000 x g, 10 minutes), and then plasmid DNA was prepared using a QIAprep Spin Miniprep (Qiagen, Crawley, UK) according to manufacturer's instructions. Cells were lysed in alkaline sodium dodecyl sulphate (SDS) solution, and then unwanted material was precipitated through addition of acid solution. This material was sedimented by centrifugation (12000 x g, 10 minutes), and the plasmid DNA in the supernatant was then bound to an anion exchange column, washed, and eluted in 30μl distilled water. Plasmid DNAs were analysed through restriction digestion and agarose gel electrophoresis (see above).

For preparation of larger plasmid preparations for transfection of PC12 cells, single clones or 1ml cultures (left over from cultures used for minipreps above) were used to inoculate 150ml LB medium containing an appropriate antibiotic, and these cultures were then grown overnight at 37°C with shaking at 250RPM. Plasmid DNA was purified from the resulting stationary cultures using QIAfilter Plasmid Maxi kits (Qiagen) according to manufacturer's instructions. DNA concentration in the preparations produced was quantified through spectrophotometry.

2.1.8 Gateway LR reaction

In order to facilitate rapid subcloning of a given coding sequence into multiple vectors, several sequences were initially inserted into the pENTR2A 'entry' vector by the conventional techniques described above. Within this vector, sequences are flanked by *att*L1 and *att*L2 attachment sites, and can be subcloned into pDEST 'destination' vectors carrying *att*R1 and *att*R2 attachment sites by enzyme-mediated transposition (Landy, 1989). Such transposition reactions are the basis for the Gateway system (Invitrogen, Paisley, UK). In LR transposition reactions, 300ng of each pENTR2A clone and pDEST destination vector were mixed with 2μl LR Clonase II (Invitrogen)(a propriatory mixture of integrase, excisionase and host integration factor) with distilled water to a total volume of 10μl. Reactions were incubated at room temperature for 3 hours and then incubated with 1μl proteinase K (2mg/ml) at 37°C for 15 minutes in order to inactivate the enzymes. 5μl of each reaction was used to transform chemically competent *E. coli* (see above). Since the pENTR2A vector confers ampicillin resistance, while the pDEST vectors used here confer kanamycin resistance, it was possible to select transformant clones on this basis. pDEST vector not undergoing transposition carries the *ccd*B gene, which prevents its propagation in most strains of *E. coli*, by lethally targeting DNA gyrase (Bernard and Couturier, 1992; Bernard *et al.*, 1993).

2.1.9 Site directed mutagenesis

Site directed mutagenesis was carried out using either the Quickchange system (Stratagene, Cambridge, UK), or the GeneTailor system

(Invitrogen, Paisley, UK) according to manufacturers' instructions. In the Quickchange system, template vector was amplified by PCR using complimentary primers carrying the desired mutation(s). The template was then digested using the restriction endonuclease *Dpn*I, and the 'nicked' linear PCR products were used to transform XL-1 Blue *E. coli* (see above). In the GeneTailor system, DNA was methylated prior to PCR amplification using overlapping primers, one of which contained the desired mutation(s). The PCR products were then used to transform *E. coli* carrying wild-type *mcrBC*. The methylated template was destroyed by these cells, and so only the mutated plasmid is propagated.

PfuTurbo polymerase (Stratagene) was used in the PCR-mediated amplification of mutated vector DNA. Each 50μl reaction mixture contained 5μl 10x PCR buffer (Stratagene), 0.2mM dNTPs, 62.5ng each of the forward and reverse primers, and 1μl (2.5U) PfuTurbo polymerase in distilled water. 50ng plasmid DNA was used as template. The PCR conditions used were as follows:-

1 cycle:	95°C	30 seconds
16 cycles:	95°C	30 seconds
	55°C	1 minute
	68°C	2 minutes per kb product
1 cycle	30°C	1 minute

2.2 Plasmids

2.2.1 pEGFP-Rab3A

The rat Rab3A sequence was amplified from an existing vector (Haynes *et al.*, 2001) by PCR, and inserted, in frame, into the pEGFP-C1 vector (Clontech, Basingstoke, UK) to generate a mammalian expression construct for Rab3A, N-terminally tagged with EGFP. Primers contained restriction endonuclease sites (underlined) to facilitate subcloning. The sense primer used was 5'-AGCAGAAGCTTTAAATATGGCATCTGCCACA GACGCT-3'-myc; *Hind*III, and the antisense primer was 5'-CAAGTA TGGATCCGCTCAGCAGGCGCAGTCCT GATGCGG-3'-myc; *Bam*HI. The amplified sequence and the vector were digested with *Hind*III/*Bam*HI, and the resulting fragments were ligated using standard methods.

2.2.2 pmRFP-Rab3A

The Rab3A sequence was cut from the pEGFP-Rab3A vector and inserted, in frame, into the pmRFP-C1 vector (kind gift of R. Y. Tsien, Department of Pharmacology, University of California, San Diego, CA) to generate a mammalian expression construct for Rab3A, N-terminally tagged with mRFP. The fragment encoding Rab3A, and the cut pmRFP-C1 vector, were each generated through digestion with *Hind*III/*Bam*HI, and these fragments were then ligated using standard methods.

2.2.3 pECFP-Rab27A

The rat Rab27A sequence was amplified from PC12 cell cDNA by PCR, and inserted in frame into the pECFP-C1 vector (Clontech, Basingstoke, UK) to generate a mammalian expression construct for Rab27A, N-terminally tagged with EGFP. Primers contained restriction endonuclease sites (underlined) to facilitate subcloning. The sense primer used was 5'-AGCAGAAGCTTTAAATA TGTCGGATGGAGATTATGAC-3'-myc; *Hind*III, and the antisense primer was 5'-CAAGTATGGATCCGCTCAACAGC CGCATAACCCCTTCTC-3'-myc; *Bam*HI. The amplified sequence and the vector were digested with *Hind*III/*Bam*HI, and the resulting fragments were ligated using standard methods.

2.2.4 pECFP-Rab27A(Q78L)

The ECFP-tagged Rab27A(Q78L) mutant was made through substitution of Leu for Gln at residue 78 using pECFP-Rab27A as a template and the primer pair 5'-GTTATGGGACACGGCGGGGCTGGAGAGGTTTCGTAG CCTG-3' (sense) and 5'-CAGGCTACGAAACCTCTCCAGCCCCGCCG TGTCCCATAAC-3' (antisense). Mutants were generated using the Quickchange system (Stratagene, Cambridge, UK) according to manufacturer's instructions.

2.2.5 pECFPRab27A(N133I)

The ECFP-tagged Rab27A(N133I) mutant was made through substitution of Ile for Asn at residue 133 using pECFP-Rab27A as a template and the primer pair 5'-GATATAGTGCTCTGCGGAATTAAGAGTGACCTAGAA

GAC-3' (sense) and 5'-GTCTTCTAGGTCACTCTTAATTCCGCAGAGCAC TATATC-3' (antisense). Mutants were generated using the Quickchange system (Stratagene, Cambridge, UK) according to manufacturer's instructions.

2.2.6 pEGFP-Rab27A

The Rab27A sequence was cut from the pECFP-Rab27A vector and inserted, in frame, into the pEGFP-C1 vector (Clontech, Basingstoke, UK) to generate a mammalian expression construct for Rab27A, N-terminally tagged with EGFP. The fragment encoding Rab27A, and the cut pEGFP-C1 vector, were each generated through digestion with *Hind*III/*Bam*HI, and these fragments were then ligated using standard methods.

2.2.7 pmRFP-Rab27A

The Rab27A sequence was cut from the pECFP-Rab27A vector and inserted, in frame, into the pmRFP-C1 vector to generate a mammalian expression construct for Rab27A, N-terminally tagged with mRFP. The fragment encoding Rab27A, and the cut pmRFP-C1 vector, were each generated through digestion with *Hind*III/*Bam*HI, and these fragments were then ligated using standard methods.

2.2.8 pRabphilinSTOP pENTR2A

The rat Rabphilin sequence was amplified from PC12 cell cDNA by PCR, and inserted into the pENTR2A (kind gift of J Johnson, Department of Physiology, University of Liverpool, UK) vector to generate a Gateway

'entry vector' construct for Rabphilin, carrying a stop codon at the 3' end of the insert. Primers contained restriction endonuclease sites (underlined) to facilitate subcloning. The sense primer used was 5'-AGCCTGTCGAC ATGACTGACACTGTGGTGAACCGTTG-3'-myc; *SalI*, and the antisense primer was 5'-AGGGCGCGGCCGCCTAGTCGCTCGACACGTGGTTCT CGT-3'-myc; *NotI*. The amplified sequence and the vector were digested with *SalI/NotI*, and the resulting fragments were ligated using standard methods.

2.2.9 pRabphilin pENTR2A

The rat Rabphilin sequence was amplified from PC12 cell cDNA by PCR, and inserted into the pENTR2A vector to generate a Gateway 'entry vector'. Primers contained restriction endonuclease sites (underlined) to facilitate subcloning. The sense primer used was 5'- AGCCTGTCGA CATGACTGACACTGTGGTGAACCGTTG-3'-myc; *SalI*, and the antisense primer was 5'- GCGCCGCGGCCGCCTAGTCGCTCGACACGTGGTTCT CGTTCT-3'-myc; *NotI*. The amplified sequence and the vector were digested with *SalI/NotI*, and the resulting fragments were ligated using standard methods.

2.2.10 pEGFP-Rabphilin

The rat Rabphilin sequence was transposed from pRabphilinSTOP pENTR2A, to pDEST-EGFP-C1 (kind gift of J. Johnson, Department of Physiology, University of Liverpool, UK) through the Gateway LR reaction.

2.2.11 pRabphilin-EGFP

The rat Rabphilin sequence was transposed from pRabphilin pENTR2A, to pDEST-EGFP-N1 (kind gift of J. Johnson, Department of Physiology, University of Liverpool, UK) through the Gateway LR reaction.

2.2.12 pRabphilin pcDNA3.1(+)

The rat Rabphilin sequence was transposed from pRabphilinSTOP pENTR2A, to pDEST-cDNA3.1(+) (kind gift of J. Johnson, Department of Physiology, University of Liverpool, UK) through the Gateway LR reaction.

2.2.13 pEGFP-Rabphilin(1-206)

The EGFP-tagged Rabphilin(1-206) mutant was made through substitution of the 207th codon, AGG, for a TGA stop codon using pEGFP-Rabphilin as a template and the primer pair 5'-GACATGGAGGACAGGTGAG CCCCAGGAC-3' (sense) and 5'-CCTGTCCTCCATGTCACCTCGAGCT-3' (antisense). Mutants were generated using the GeneTailor system (Invitrogen, Paisley, UK) according to manufacturer's instructions.

2.2.14 pEGFP-Rabphilin(AAA)

The EGFP-tagged Rabphilin(AAA) mutant was made through substitution of condons for Trp-Phe-Phe, for codons for Ala-Ala-Ala, at residues 153-155 using pEGFP-Rabphilin as a template and the primer pair 5'-AAGCGCTCGGGAGCAGCGGCTGCTAAGGGT TTCC-3' (sense) and 5'-TGCTCCCGAGCGCTTCCAGACCTCT-3' (antisense). Underscoring reflects point mutations in the template sequence. Mutants were generated

using the GeneTailor system (Invitrogen, Paisley, UK) according to manufacturer's instructions.

2.2.15 pGranuphilinSTOP pENTR2A

The rat Granuphilin sequence was amplified from PC12 cell cDNA by PCR, and inserted into the pENTR2A vector to generate a Gateway 'entry vector' construct for Granuphilin, carrying a stop codon at the 3' end of the insert. Primers contained restriction endonuclease sites (underlined) to facilitate subcloning. The sense primer used was 5'-TACATGTCGACATGTCGGAGATACTAGACCTCTCTTT-3'-myc; *SalI*, and the antisense primer was 5'-TACATGCGGCCGCTCATACACCCAGC TTCTGCTTGACCA-3'-myc; *NotI*. The amplified sequence and the vector were digested with *SalI/NotI*, and the resulting fragments were ligated using standard methods.

2.2.16 pGranuphilin pENTR2A

The rat Granuphilin sequence was amplified from PC12 cell cDNA by PCR, and inserted into the pENTR2A vector to generate a Gateway 'entry vector' construct. Primers contained restriction endonuclease sites (underlined) to facilitate subcloning. The sense primer used was 5'-TACATGTCGACATGTCGGAGATACTAGACCTCTCTTT-3'-myc; *SalI*, and the antisense primer was 5'- TACATGCGGCCGCTACACCCA GCTTCTGCTTGACCATTG-3'-myc; *NotI*. The amplified sequence and the vector were digested with *SalI/NotI*, and the resulting fragments were ligated using standard methods.

2.2.17 pEGFP-Granuphilin

The rat Granuphilin sequence was transposed from pGranuphilinSTOP pENTR2A, to pDEST-EGFP-C1 through the Gateway LR reaction.

2.2.18 pGranuphilin-EGFP

The rat Granuphilin sequence was transposed from pGranuphilin pENTR2A, to pDEST-EGFP-N1 through the Gateway LR reaction.

2.2.19 pGranuphilin pcDNA3.1(+)

The rat Granuphilin sequence was transposed from pGranuphilinSTOP pENTR2A, to pDEST-cDNA3.1(+) through the Gateway LR reaction.

2.2.20 pSlp5STOP pENTR2A

The rat Slp5 sequence was amplified from PC12 cell cDNA by PCR, and inserted into the pENTR2A vector to generate a Gateway 'entry vector' construct for Slp5, carrying a stop codon at the 3' end of the insert. Primers contained restriction endonuclease sites (underlined) to facilitate subcloning. The sense primer used was 5'-TACATGTCGACATG TCTAAGAACTCGGAGTTCATCAA-3'-myc; *SalI*, and the antisense primer was 5'-TACATGCGGCCGCTCAGAGCCTACATTTCGCCATGCTAG-3'-myc; *NotI*. The amplified sequence and the vector were digested with *SalI/NotI*, and the resulting fragments were ligated using standard methods.

2.2.21 pSlp5 pENTR2A

The rat Slp5 sequence was amplified from PC12 cell cDNA by PCR, and inserted into the pENTR2A vector to generate a Gateway 'entry vector' construct. Primers contained restriction endonuclease sites (underlined) to facilitate subcloning. The sense primer used was 5'- TACATGTCGAC ATGTCTAAGAACTCGGAGTTCATCAA-3'-myc; *SalI*, and the antisense primer was 5'-TACATGCGGCCGCGAGCCTACATTTCGCCATGCTAGA AC-3'-myc; *NotI*. The amplified sequence and the vector were digested with *SalI/NotI*, and the resulting fragments were ligated using standard methods.

2.2.22 pEGFP-Slp5

The rat Slp5 sequence was transposed from pSlp5STOP pENTR2A, to pDEST-EGFP-C1 through the Gateway LR reaction.

2.2.23 pSlp5-EGFP

The rat Slp5 sequence was transposed from pSlp5 pENTR2A, to pDEST-EGFP-N1 through the Gateway LR reaction.

2.2.24 pSlp5 pcDNA3.1(+)

The rat Slp5 sequence was transposed from pSlp5STOP pENTR2A, to pDEST-cDNA3.1(+) through the Gateway LR reaction.

2.2.25 pNoc2-EYFP

The Noc2 sequence was amplified from pNoc2 (Haynes *et al.*, 2001) and inserted in frame into the pEYFP-N1 vector (Clontech, Basingstoke, UK) to generate a mammalian expression construct for Noc2, C-terminally tagged with EYFP. Primers contained restriction endonuclease sites (underlined) to facilitate subcloning. The sense primer used was 5'-TGACAAGCTT ATGGCTGACACCATCTTCAGCAG-3'-myc; *Hind*III, and the antisense primer was 5'-TGACCCGCGGGTAATGGGTAGTTGCCCAG-3'-myc; *Sac*II. The amplified sequence and the vector were digested with *Hind*III/*Sac*II, and the resulting fragments were ligated using standard methods.

2.2.26 pNoc2(V58A)-EYFP

A Noc2 sequence carrying a point mutation leading to substitution of Ala for Val at residue 58 was amplified from pNoc2(V58A) (Haynes *et al.*, 2001). This was then inserted in frame into the pEYFP-N1 vector (Clontech, Basingstoke, UK) to generate a mammalian expression construct for Noc2(V58A), C-terminally tagged with EYFP. Primers contained restriction endonuclease sites (underlined) to facilitate subcloning. The sense primer used was 5'-TACTAGGCTAGCA ATGGCTGACACCATCTTCAGCAG-3'-myc; *Nhe*I, and the antisense primer was 5'-CAG TTGCCCGGGCGTAATGGGTAGTTGCCCAG-3'-myc; *Xma*I. The amplified sequence and the vector were digested with *Nhe*I/*Xma*I, and the resulting fragments were ligated using standard methods.

2.2.27 pNoc2(AAA)-EYFP

A Noc2 sequence carrying point mutations leading to substitution of Ala residues for Trp, Phe and Tyr at residues 154-156 was amplified from pNoc2(AAA) (Haynes *et al.*, 2001). This was then inserted in frame into the pEYFP-N1 vector (Clontech, Basingstoke, UK) to generate a mammalian expression construct for Noc2(AAA), C-terminally tagged with EYFP. Primers contained restriction endonuclease sites (underlined) to facilitate subcloning. The sense primer used was 5'-TACTAGGCTAGCAAT GGCTGACACCATCTTCAGCAG-3'-myc; *Nhe*I, and the antisense primer was 5'-CAGTTGCCCGGGCGTAATGGGTAGTTGCCCAG-3'-myc; *Xma*I. The amplified sequence and the vector were digested with *Nhe*I/*Xma*I, and the resulting fragments were ligated using standard methods.

2.2.28 pNoc2-EGFP

The Noc2 sequence was cut from the pNoc2-EYFP vector and inserted, in frame, into the pEGFP-N1 vector (Clontech, Basingstoke, UK) to generate a mammalian expression construct for Noc2, C-terminally tagged with EGFP. The fragment encoding Noc2, and the cut pEGFP-C1 vector, were each generated through digestion with *Hind*III/*Sac*II, and these fragments were then ligated using standard methods.

2.2.29 Sequencing

Sequences contained in plasmids generated through cloning or site-directed mutagenesis were verified by automatic sequencing carried out by DBS Genomics (Durham, UK) or 'The Sequencing Service' (Dundee, UK).

2.2.30 Other Plasmids

The plasmids tabulated below were constructed elsewhere as described:-

Plasmid	Reference
pArf1-EGFP	As Arf-1-ECFP in (Haynes *et al.*, 2005)
pECFP-C1	Clontech, Basingstoke, UK
pEGFP-C1	Clontech, Basingstoke, UK
pEGFP-N1	Clontech, Basingstoke, UK
pEYFP-N1	Clontech, Basingstoke, UK
pGCR-EGFP	J Johnson, Liverpool, UK
pMunc18-1	(Ciufo *et al.*, 2005)
pMunc18-1(E466K)	(Ciufo *et al.*, 2005)
pMunc18-1(E466K/R39C)	(Ciufo *et al.*, 2005)
pNoc2	(Haynes *et al.*, 2001)
pppANF-EGFP	(Burke *et al.*, 1997)
pXGH5	Nichols Institute Diagnostics, (Selden *et al.*, 1986)

Plasmids encoding putative RabGAPs carrying myc-tags were the kind gift of Professor F Barr (Liverpool, UK). These were as follows:-

pRabGAP-5-myc; pParis1-myc; pRab3GAP-myc; pRUPTBC2-myc; pGAPCenA-myc; pGyp6-myc; pUSP6-myc; pTBC1D22B-myc; pTBC1D1-myc; pTBC1D16-myc; pEVI5-myc; pRN-tre-myc; pRUPTBC1-myc; pTBC1D3-myc; pTBC1D7-myc; pTBC1D10A-myc; pTBC1D10B-myc; pTBC1D10C-myc; pTBC1D12-myc; pTBC1D13-myc; pTBC1D14-myc; pTBC1D15-myc; pTBC1D17-myc; pTBC1D18-myc; pTBC1D19-myc; pTBC1D20-myc; pTBC1D21-myc; pGyp1-myc; pAK074305-myc; pAAH_33712-myc; pKIAA0882-myc; pNM_018309-myc; pEvi5-like-myc; pXM_037557-myc; pXM_370928-myc; pNM_198868-myc; pTBC1D4-myc.

2.3 Cell culture and transfection

PC12 cells were grown in suspension in 75cm^2 culture flasks at 37°C, 5% CO_2 in RPMI 1640 medium (Gibco, Paisley, UK) supplemented with 10% horse serum, 5% foetal calf serum, 100U/ml penicillin and 0.1mg/ml streptomycin. Cells were passaged once weekly.

Transfection of PC12 cells was carried out as follows. Coverslips in live-cell dishes, 6 or 24-well plates, were seeded with ~3 x 10^5 cells/lml supplemented media (as above). To facilitate cells' adherence, cover slips were coated with collagen VII, or poly-D-lysine (Sigma Aldrich, Dorset, UK). In some cases, commercial BD Biocoat^{TM} poly-D-lysine-coated coverslips were used (VWR, Dorset, UK). For hGH cotransfection assays, ~3 x 10^5 cells were seeded onto commercial BD Biocoat^{TM} poly-D-lysine-coated plates (VWR). In each case, cells became adherent following incubation overnight at 37°C, 5% CO_2. Before transfection, media was removed, and replaced with 400-1400µl/well Optimem 1 media (Gibco, Paisley, UK). Transfection mixes were made up with 3µl of the Lipofectamine 2000 reagent (Invitrogen, Paisley, UK) per µg plasmid in 100µl Optimem 1 according to manufacturer's instructions. Mixes were incubated at room temperature for 30 minutes in order for the lipid and the DNA to associate and then added dropwise to the cells. 5 hours following their addition, the transfection mixes were removed from cells and replaced with supplemented media. Cells were incubated for a further 18-54 hours prior to experiments.

The following table indicates the levels of each plasmid used in the experiments shown in the figures presented in this study:-

Figure	Plasmid(s)	ng per transfection
3.1A-C	pEGFP-Rab3A	500
3.1D-F	pEGFP-Rab3A	500
	pECFP-Rab27A	500
3.2A-C	pECFP-Rab27A	500
3.3A-C, J-L	pppANF-EGFP	500
3.3D-F, M-O	pEGFP-Rab3A	500
3.3G-I, P-R	pEGFP-Rab27A	500
3.3T-V	pECFP-Rab27A	500
	pEGFP-Rab3A	500
3.4A, D	pppANF-EGFP	500
3.4B, E	pEGFP-Rab3A	500
3.4C, F	pEGFP-Rab27A	500
3.4G (top)	pmRFP-Rab3A	500
	pppANF-EGFP	500
3.4G (bottom)	pmRFP-Rab27A	500
	pppANF-EGFP	500
3.5A	pXGH5	1000
	pEGFP-C1	As indicated
	pEGFP-Rab3A	As indicated
3.5B	pECFP-C1	As indicated
	pECFP-Rab27A	As indicated
3.6A, D	pECFP-Rab27A	500
3.6B, E	pECFP-Rab27A(Q78L)	500
3.6C, F	pECFP-Rab27A(N133I)	500
3.6G	pXGH5	1000
	pECFP-C1	As indicated
	pECFP-Rab27A	As indicated
	pECFP-Rab27A(Q78L)	As indicated
	pECFP-Rab27A(N133I)	As indicated
3.7A-D	pArf-1-EGFP	500
3.7E-H	pppANF-EGFP	500
3.7I-L	pEGFP-Rab3A	500
3.7M-P	pEGFP-Rab27A	500
3.7S	pppANF-EGFP	500
3.8	pEGFP-Rab3A	500
3.9B	pXGH5	1000
3.9C-D	pGCR-EGFP	500
3.10A	pppANF-EGFP	100
3.10B-C (top)	pppANF-EGFP	100
3.10B-C (middle)	pEGFP-Rab3A	100
3.10B-C (bottom)	pEGFP-Rab27A	100
4.1A	pRabphilin-EGFP	1000
4.1B	pEGFP-Rabphilin	1000
4.1C	pGranuphilin-EGFP	1000
4.1D	pEGFP-Granuphilin	1000
4.1E	pSlp5-EGFP	1000
4.1F	pEGFP-Slp5	1000
4.1G	pNoc2-EGFP	1000
4.2A-C	pEGFP-Rabphilin	500
	pmRFP-Rab3A	500
4.2D-F	pGranuphilin-EGFP	500
	pmRFP-Rab3A	500
4.2G-I	pNoc2-EGFP	500
	pmRFP-Rab3A	500
4.2J-L	pEGFP-Rabphilin	500
	pmRFP-Rab27A	500
4.2M-O	pGranuphilin-EGFP	500
	pmRFP-Rab27A	500
4.2P-R	pNoc2-EGFP	500
	pmRFP-Rab27A	500
4.3A	pXGH5	500
	pEGFP-C1	As indicated
	pRabphilin pcDNA3.1(+)	As indicated
	pGranuphilin pcDNA3.1(+)	As indicated
	pSlp5 pcDNA3.1(+)	As indicated
4.3B	pXGH5	1000
	pEGFP-N1	As indicated
	pNoc2	As indicated
4.4A-C	pECFP-Rab27A	500
	pNoc2-EYFP	500
4.4D-F	pECFP-Rab27A	500
	pNoc2(V58A)-EYFP	500
4.4G-I	pECFP-Rab27A	500
	pNoc2(AAA)-EYFP	500
4.4J	pNoc2(AAA)-EYFP	500
4.4K	pXGH5	1000
	pEYFP-N1	As indicated
	pNoc2-EYFP	As indicated

Figure	Plasmid(s)	ng per transfection
	pNoc2(V58A)-EYFP	As indicated
	pNoc2(AAA)-EYFP	As indicated
4.5A-D	pEGFP-Rabphilin	500
4.5E-H	pGranuphilin-EGFP	500
4.5I-L	pNoc2-EGFP	500
4.6A	pEGFP-Rabphilin	500
4.6B	pEGFP-Rabphilin(1-206)	500
4.6C	pEGFP-Rabphilin(AAA)	500
4.7A	pEGFP-Rab3A	200
	[+/-] pMunc18-1(E466K)	800
4.7B-C	pMunc18-1	900
	pEGFP-Rab3A	100
4.7D-E	pEGFP-Rab3A	100
4.7F	pEGFP-Rab3A	200
4.7G	pMunc18-1	800
	pEGFP-Rab3A	200
4.7H	pMunc18-1(E466K)	800
	pEGFP-Rab3A	200
4.7I	pMunc18-1(E466K/R39C)	800
	pEGFP-Rab3A	200
4.8A	pEGFP-Rab27A	200
4.8B	pMunc18-1	800
	pEGFP-Rab27A	200
4.8C	pMunc18-1(E466K)	800
	pEGFP-Rab27A	200
4.8D	pMunc18-1(E466K/R39C)	800
	pEGFP-Rab27A	200
4.8F-G (right)	pMunc18-1(E466K)	800
4.9A (bottom)	pMunc18-1(E466K)	800
5.1A-C	pEGFP-Rab3A	250
	pRab3GAP-myc	750
5.1D-F	pEGFP-Rab27A	250
	pRab3GAP-myc	750
5.1G	pXGH5	1000
	pEGFP-N1	As indicated
	pRab3GAP-myc	As indicated
5.2A-F	pEGFP-Rab27A	250
	pTBC1D10A-myc	750
5.2G-L	pEGFP-Rab3A	250
	pTBC1D10A-myc	750
5.2M	pXGH5	1000
	pEGFP-N1	As indicated
	pTBC1D10A-myc	As indicated
5.3A-F	pEGFP-Rab27A	250
	pTBC1D10B-myc	750
5.3G-L	pEGFP-Rab3A	250
	pTBC1D10B-myc	750
5.3M	pXGH5	1000
	pEGFP-N1	As indicated
	pTBC1D10B-myc	As indicated
5.4A	pTBC1D7-myc	750
5.4B	pTBC1D10C-myc	750
5.4C	pTBC1D14-myc	750
5.4D	pTBC1D19-myc	750
5.4E	pNM_018309-myc	750
5.4F	pXM_037557-myc	750
5.4G	pTBC1D3-myc	750
5.4H	pRN-tre-myc	750
5.5A-C	pEGFP-Rab3A	250
	pRUTBC1-myc	750
5.5D-F	pEGFP-Rab27A	250
	pRUTBC1-myc	750
5.5G	pXGH5	1000
	pEGFP-N1	As indicated
	pEGFP-Rab3A	As indicated
	pRUTBC1-myc	As indicated
5.5H	pXGH5	1000
	pEGFP-N1	As indicated
	pEGFP-Rab27A	As indicated
	pRUTBC1-myc	As indicated
5.6A-F	pEGFP-Rab27A	250
	pRUTBC2-myc	750
5.6G-I	pEGFP-Rab3A	250
	pRUTBC2-myc	750
5.6J	pXGH5	1000
	pEGFP-N1	As indicated
	pEGFP-Rab27A	As indicated
	pRUTBC2-myc	As indicated

2.4 Cell preparation and immunocytochemistry

2.4.1 Fixation and immunocytochemistry

Coverslips were washed in phosphate-buffered saline (PBS) and then fixed in 0.5ml 4% paraformaldehyde for 30 minutes at room temperature. Following this fixation step, the coverslips were again washed twice with PBS. Where cells were not to be immunostained, coverslips were then airdried and mounted onto slides. Where cells were to be immunostained, they were incubated in PBT (PBS containing 0.3% BSA and 0.1% Triton-X-100) for 1 hour, at room temperature, prior to the addition of primary antibody. Cells were immunostained by exposure to primary antibody in PBT for 1 hour at room temperature, or overnight at 4°C. Unbound antibody was removed by washing three times with PBT, and coverslips were then exposed to appropriate secondary antibodies. Coverslips exposed to an Alexa Fluor 488-conjugated, or an Alexa Fluor 594-conjugated secondary (Molecular Probes, Paisley, UK), were incubated for 1 hour with a 1:100 dilution of this antibody in PBT, washed twice in PBS, airdried, and mounted onto slides. Coverslips exposed to a biotinylated secondary (Amersham Biosciences, Buckinghamshire, UK) were incubated for 1 hour with a 1:100 dilution of this antibody in PBT, washed twice in PBT. They were then exposed to a 1:50 dilution of streptavidin-conjugated Texas red (Amersham Biosciences) in PBT for 30 minutes, washed and mounted as above. The following table shows the specific primary and secondary antibodies used in the experiments shown in the figures presented in this study:-

Figure	Primary Antibody	Dilution	Secondary Antibody
3.1A-C	anti-Rab3A	1:400	Biotin-anti-Mouse
	anti-Secretogranin II	1:100	Biotin-anti-Rabbit
3.2A-C	anti-CSP	1:400	Biotin-anti-Rabbit
3.3A-R	anti-Secretogranin II	1:100	Alexa 594-anti-Rabbit
4.7B	anti-Munc18	1:400	Alexa 594-anti-Rabbit
4.8F	anti-Rab3A	1:400	Alexa 488-anti-Mouse
4.9A	anti-Munc18	1:400	Alexa 594-anti-Rabbit
5.1-5.6	anti-myc	1:200	Alexa 594-anti-Rabbit

Primary antibodies were sourced as follows:-

Species	Antibody	Source
Rabbit	anti-CSP	(Chamberlain *et al.*, 1996)
Rabbit	anti-Munc18	Calbiochem, CA, USA
Rabbit	anti-myc	Sigma, Dorset, UK
Mouse	anti-Rab3A	Transduction Labs, Cowley, UK
Rabbit	anti-Secretogranin II	Abcam, Cambridge, UK

2.4.2 Temperature-block experiments

Incubation of PC12 cells at 20°C prevents vesicle formation at the trans-Golgi network (TGN)(Rudolf *et al.*, 2001). This phenomenon was exploited in experiments used to examine association of proteins with newly synthesised and immature secretory granules. PC12 cells were transfected as normal (see above), but following 2 hours incubation at 37°C, 5% CO_2, they were incubated for a further 16 hours at 20°C. They were then incubated at 37°C, 5% CO_2, for a measured time of between 0-30 minutes, after which they were immediately fixed as described above.

2.4.3 Digitonin permeablisation

Digitonin permeablisation was used to remove cytosolic protein from cells prior to fixation. Coverslips were washed three times in Krebs-Ringer

solution (145mM NaCl, 5mM KCl, 1.3mM $MgCl_2$, 1.2mM NaH_2PO_4, 10mM Glucose, 20mM HEPES; pH7.4) and then incubated for 15minutes with 20µM digitonin in a permeablisation buffer containing 139mM potassium glutamate, 20mM PIPES, 5mM EGTA, 2mM $MgCl_2$ and 2mM ATP (pH6.5). Cells were then immediately fixed and mounted as described above.

2.4.4 Phalloidin staining

After fixation cells were incubated with anti-Munc18-1 and actin filaments were stained with 25nM TRITC-conjugated Phalloidin (Molecular Probes, Paisley, UK) which was visualised with a 543nm laser and light collected between 500-535nm.

2.5 Confocal microscopy

2.5.1 Fixed-cell imaging

Fixed-cell imaging was carried out on a Leica TCS-SP microscope (Leica Microsystems, Heidelberg, Germany). A 63x oil immersion objective with a 1.4 numerical aperture was used, and the pinhole was set to airy1. ECFP-containing constructs were excited using a 405nm laser, and emitted light was collected at 450-500nm. EGFP-containing constructs and Alexa Fluor-488 were excited using a 488nm laser, and light was collected at 500-550nm. EYFP-containing constructs were excited using a 514nm laser, and light was collected at 525-575nm. mRFP-containing constructs, Texas Red, and Alexa Fluor 594 were excited using a 593nm laser, and light was collected at 625-675nm.

2.5.2 Quantification of fluorescence distribution

In order to quantify the effects of cooverexpression of one protein, on the peripheral distribution of another within fixed cells, the following means of quantification was developed. Regions of interest were drawn around the outside of each cell, immediately beneath the cell periphery, and around the nucleus. Peripheral fluorescence was calculated by subtraction of cytoplasmic and nuclear fluorescence from the outer region of interest. Cytosolic fluorescence was calculated by subtraction of nuclear fluorescence. Ratios of peripheral to cytosolic fluorescence were calculated on the basis of the mean fluorescence per pixel in respective

regions rather than total fluorescence to avoid problems arising from variations in the size of the selected regions of interest.

2.5.3 Quantification of glucocorticoid receptor translocation

In order to quantify the effects of the heat-shock protein 90 (HSP90) inhibitor geldanamycin (GA) on the dexamethasone-induced translocation of glucocorticoid receptor (GCR)-EGFP to PC12 cell nuclei, the following protocol was taken from a previous study (Galigniana *et al.*, 1998). 18 hours following transfection with GCR-EGFP, control cells were chilled on ice, and exposed to 0μM or 1μM dexamethasone for 1 hour, while treated cells were exposed to 1μM dexamethasone for 30 minutes, and then a combination of 1μM dexamethasone and 10μM GA for 30 minutes. Solutions were made in Krebs Ringer buffer (NaCl 145mM, HEPES 20mM, Glucose 10mM, KCl 5mM, $MgCl_2$ 1.3mM, NaH_2PO_4 1.2Mm). Cells were fixed following 20 minutes incubation at 37°C, 5% CO_2, and 100 cells per condition were imaged. GCR-EGFP localisation in each cell was blindly scored as either predominantly nuclear or cytoplasmic, and this data was then used to calculate cytoplasmic:nuclear ratios for each condition.

2.5.4 Live-cell imaging

Live-cell imaging was carried out on a Leica TCS-SP-MP microscope (Leica Microsystems, Heidelberg, Germany). A 63x water immersion objective with a 1.2 numerical aperture was used, and images were captured every 1.64 seconds. Cells were maintained at room temperature. EGFP-containing constructs were excited using a 488nm laser, and light

was collected at 500-550nm. X-Rhod was excited using a 593nm laser, and light was collected at 600-650nm.

2.5.5 Fluorescence recovery after photobleaching

In order to investigate the dynamics of EGFP-containing constructs in live cells, fluorescence recovery after photobleaching (FRAP) was used. PC12 cells, imaged as above, were imaged at a 3x zoom, and the confocal pinhole was set to airy 2.06. Bleaching was carried out using the FRAP macro and 'zoom-in' functions available on the Leica confocal software. Circular regions of interest, of diameter 2.5µm, were bleached using 75% of maximal laser power. To determine the rate of fluorescence recovery, fluorescence in these regions was measured over time, and then normalised with respect to corresponding total cellular fluorescence at each individual time point, to correct for bleaching during low level imaging. 10 pre-bleach and 100 post-bleach frames were recorded per cell. Cells were maintained in Krebs Ringer buffer (NaCl 145mM, HEPES 20mM, Glucose 10mM, KCl 5mM, $MgCl_2$ 1.3mM, NaH_2PO_4 1.2Mm). Where the effects of geldanamycin (GA) were analysed, control cells were incubated in Krebs, and treated cells were incubated in Krebs containing 10µM GA, for 1 hour prior to bleaching.

2.5.6 ATP stimulation of live-cells

For characterisation of the effects of ATP stimulation on numbers of secretory granules labeled with EGFP-containing constructs in live cells, a perfusion system was used. Cells were imaged as in 'Live-cell imaging'

above, at a 6x zoom, and the confocal pinhole was set to airy 3. Transmitted light images were taken during experiments to ensure that changes in fluorescence were not the result of drift in the focal plane. Cells were maintained in Krebs Ringer buffer, containing 3mM $CaCl_2$, and were stimulated through perfusion with 300µM ATP, also in Ca^{2+}-Krebs. 10 pre-stimulation and 90 post-stimulation frames were recorded per cell. Where cells were loaded with the calcium indicator X-Rhod (Molecular Probes, Paisley, UK), they were incubated in a solution of 5µM X-Rhod in 3mM Ca^{2+}-Krebs for 20 minutes, and then in 3mM Ca^{2+}-Krebs for a further 20 minutes prior to imaging.

In order to identify any change in the numbers of granules labeled with a given EGFP-tagged construct, before and after ATP-stimulation, 'before' and 'after' frames for each cell were blindly scored for granule numbers, and mean change in granule numbers was later calculated. Frames recorded 32 seconds and 127 seconds into the stimulation protocol were used for this quantitative analysis, as these timepoints correspond to a point before the start of ATP-stimulated calcium-influx, and a point of peak intracellular calcium, in the majority of cells, as identified in terms of X-Rhod fluorescence.

2.6 hGH cotransfection assays

In order to determine the effects of coexpressed protein, or drug treatment, on secretion of exogenous human growth hormone (hGH) from PC12 cells, the following previously described protocol was used (Graham *et al.*, 2000). 48 hours following transfection (described in Section 2.3, above), cells were washed once in Krebs Ringer buffer (NaCl 145mM, HEPES 20mM, Glucose 10mM, KCl 5mM, $MgCl_2$ 1.3mM, NaH_2PO_4 1.2mM), containing 3mM $CaCl_2$, in order to remove hGH secreted prior to stimulation. Each well was then exposed to 200µl 3mM Ca^{2+}-Krebs (unstimulated) or 200µl 3mM Ca^{2+}-Krebs, 300µM ATP, (stimulated) for 15 minutes at room temperature. The hGH released in this period was quantified using an enzyme-linked immunosorbent assay (ELISA) kit (Roche, East Sussex, UK) according to manufacturer's instructions. Remaining cellular hGH was determined following lysis of cells in 200µl 0.5% Triton-X-100. hGH secretion in each well was calculated as percentage of total hGH, and presented as mean ± s.e.m. for each condition. Where the effects of geldanamycin (GA) on secretion were analysed, control cells were incubated with Ca^{2+}-Krebs, and treated cells were incubated with Ca^{2+}-Krebs containing 10µM GA, for 1 hour prior to stimulation, at room temperature.

Chapter 3:

Rab proteins in neuroendocrine exocytosis

3.1 Introduction

A broad range of cell types including neuronal, exocrine, endocrine and immune cells are specialised for regulated exocytosis, and there are sometimes dramatic differences in the regulation of the process and in the morphology of the organelles involved (Burgoyne and Morgan, 2003). It is thought that the core machinery of exocytosis, and indeed that of membrane fusion in general, is conserved in the form of the soluble N-ethyl maleimide-sensitive attachment protein receptor (SNARE) proteins, while many other proteins, including the Rabs and their effectors, are involved in adapting the process according to its discrete cellular or physiological roles. Indeed, the expansion of the Rab gene family in the higher eukaryotes (from 11 in yeast to over 60 in mammals) is likely to reflect the increased complexity and regulation of membrane trafficking in these organisms, and particularly that associated with specialisation (Bock *et al.*, 2001).

As previously described, Rabs are 'molecular switches', present in either GDP- or GTP-bound forms. When membrane-associated, they may become GTP-bound through interaction with specific guanine nucleotide exchange factors (GEFs). It is generally the GTP-bound form of the Rab that is considered active and that interacts with effectors. Rabs hydrolyse bound GTP to GDP following interaction with specific GTPase-activating proteins (GAPs). In the GDP-bound form, Rabs may be vulnerable to extraction to the cytosol, a process likely to involve guanine nucleotide

dissociation inhibitor (GDIs) since all cytosolic Rab protein is GDI-bound (Luan *et al.*, 1999). GDIs are also thought to be important for correct targeting of Rabs back onto specific target membranes, a process also hypothesised to require a GDI displacement-factor (GDF) (Pfeffer, 2005b). Standard models suggest that Rabs follow continuous cycles of organelle association and disassociation (Grosshans *et al.*, 2006). Thus, the dynamics underlying recruitment of these proteins to those target membranes are likely to be functionally important.

Recent work has established that Rab3A and Rab27A are likely to be the only two Rabs that contribute in a non-redundant manner to secretory granule exocytosis in PC12 cells (Pereira-Leal *et al.*, 2001; Fukuda, 2003a; Tsuboi and Fukuda, 2006a). This would suggest that these cells are an ideal system in which to investigate the discrete functions of these Rabs. Since little was known about the dynamic behaviour of these proteins in this cell type, this study was initially directed towards characterising that behaviour.

3.2 Results

3.2.1 Recruitment of exocytotic Rab proteins to secretory granules

In order to pursue a study focusing on the involvement of Rab proteins in exocytosis, it was initially necessary to identify a model system suited to this study. PC12 cells are derived from rat phaeochromocytoma, a tumour of adrenal chromaffin cells (Greene and Tischler, 1976). They are a very widely used cell-line in the exocytosis field since they retain the capacity for secretion and are readily-transfectable. Of relevance to this study, is that the molecular machinery of exocytosis identified in PC12 cells shares a number of characteristics with that identified in neuronal cells as well as with that identified in endocrine and exocrine cells (Burgoyne and Morgan, 2003). Of particular relevance, is that PC12 cells have been found to endogenously express Rab3A, which has been widely studied in the context of neuronal exocytosis of synaptic vesicles, and Rab27A, which has been widely studied in the context of endocrine and exocrine secretion of secretory granules (Izumi *et al.*, 2003; Sudhof, 2004). The choice of this cell-line as a model system therefore enabled simultaneous and physiologically meaningful study of both of these important proteins.

Endogenous Rab3A and Rab27A are reported to associate with secretory granules in PC12 cells (Darchen *et al.*, 1995; Tsuboi and Fukuda, 2006a). To confirm the localisation of Rab3A in the PC12 cells used in this laboratory, fixed cells were probed with antibodies against Rab3A, and the secretory granule marker protein secretogranin II (SGII) (Fig.3.1, A-C)

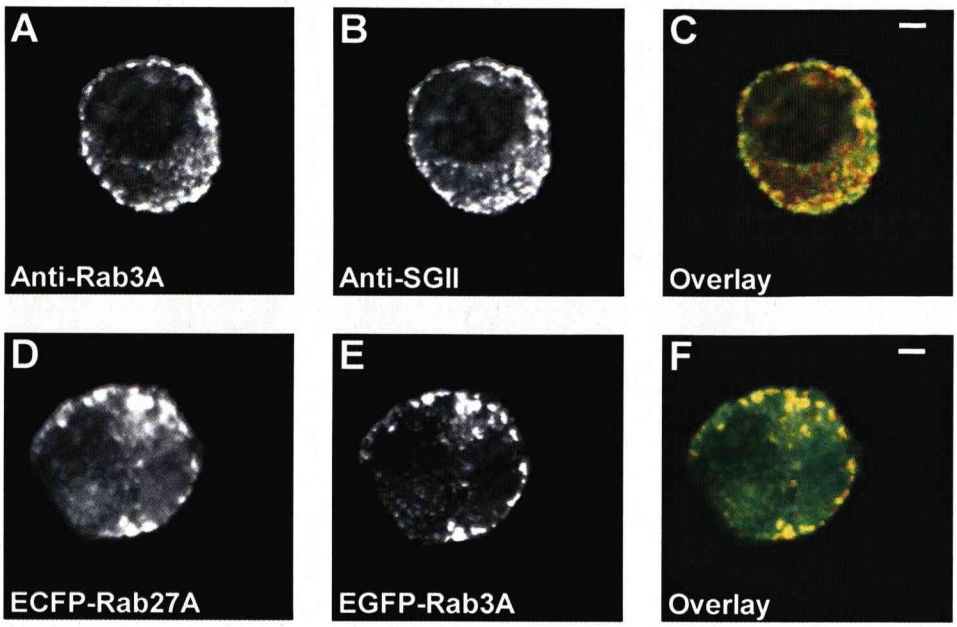


Figure 3.1. Endogenous Rab3A and secretogranin II (SGII), and exogenous ECFP-Rab27A and EGFP-Rab3A, colocalise strongly in PC12 cells. (A-C) Fixed cells co-immunostained with anti-Rab3A and anti-SGII. (D-F) Cells fixed 18 hours after cotransfection with plasmids encoding ECFP-Rab27A and EGFP-Rab3A. Each overlay image on the right of the figure is a composite of green from the left image and red from the centre image. Areas of overlap appear in yellow. Bars, 4μm.

(Steiner *et al.*, 1989). As expected, these proteins colocalised strongly at punctae within the cytosol and at the periphery of cells, consistent with their granular localisation. Unfortunately, it was not possible to carry out a similar experiment to determine the localisation of endogenous Rab27A because a suitable antibody was not available.

As tools to study the localisation and dynamics of Rab3A and Rab27A in live cells, expression vectors for N-terminally fluorescently tagged versions of these proteins were generated. N-terminal modification does not appear to affect Rab protein function, because chimeric Rab27A N-terminally tagged with GFP has the capacity to functionally replace the wild-type protein in a mouse 'knock-in' model (Tolmachova *et al.*, 2004).

Since it had not been possible to determine the localisation of endogenous Rab27A in PC12 cells in this study through immunocytochemistry, the localisation of ECFP-Rab27A with respect to that of EGFP-Rab3A was explored. When ECFP-Rab27A and EGFP-Rab3A were coexpressed, their localisation was punctate, closely resembled that of endogenous Rab3A, and the two tagged Rab proteins showed very strong colocalisation (Fig.3.1, D-F). These data were consistent with the suggestion that both constructs localise to secretory granules. In order to test this suggestion directly, the localisation of the exogenous Rabs was compared to that of secretory granule marker proteins in single cells. Examples of these experiments include comparison of ECFP-Rab27A fluorescence with staining for the granule marker cysteine string protein (CSP) (Chamberlain *et al.*, 1996) (Fig.3.2), and comparison of EGFP-Rab3A and EGFP-Rab27A fluorescence with staining for SGII (Fig.3.3, D-F and G-I respectively). Surprisingly, the localisation of the exogenous Rabs only partially matched that of the markers. By inference, this suggests that the localisation of tagged-Rab3A and that of tagged-Rab27A does not entirely match that of their endogenous counterparts.

The above might suggest that the exogenously expressed Rab proteins were mislocalised. However, in a previously presented model (Fischer von Mollard *et al.*, 1991; Sudhof, 1997), neuronal Rab3A is recruited to, and remains associated with, synaptic vesicles, until an exocytotic signal leads to fusion between the vesicular and plasma membranes; an event associated with the dispersal of the Rab (Fischer von Mollard *et al.*, 1991;

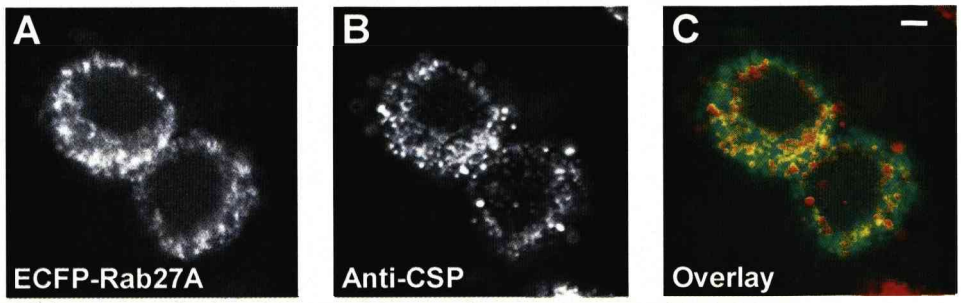
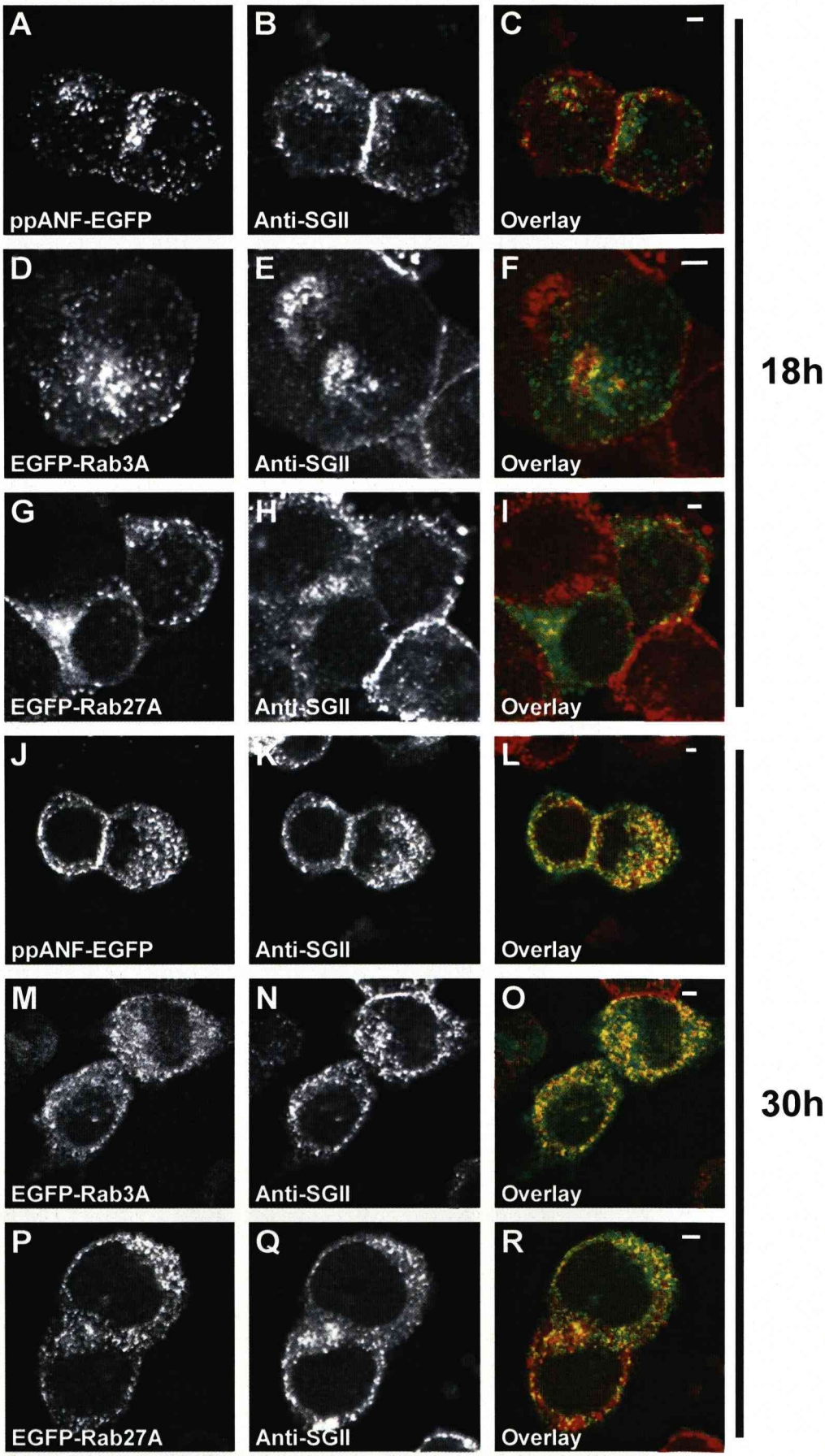


Figure 3.2. ECFP-Rab27A partially colocalises with the secretory granule marker cysteine-string protein (CSP) in PC12 cells. (A-C) Cells fixed 18 hours after transfection with a plasmid encoding ECFP-Rab27A were immunostained with anti-CSP. The overlay image on the right of the figure is a composite of green from the left image and red from the centre image. Areas of overlap appear in yellow. Bar, 4μm.

Star *et al.*, 2005). Coupled to the suggestion that Rab3A recruitment is saturable, and dependent on a proteinaceous receptor (Chou and Jahn, 2000), another possibility arises. It is possible that the newly synthesised Rabs are preferentially recruited to newly made secretory granules, not already saturated with the protein, and therefore that the tagged Rab proteins localised with only part of the cellular compliment of granules because the protein synthesised post-transfection was only recruited to secretory granules synthesised post-transfection.

To test this hypothesis, a transfectable granule content marker, prepro-atrial natriuretic factor-EGFP (ppANF-EGFP), was used. ppANF-EGFP is packaged within the granule core, and so the granules labeled by this construct are only those formed post-transfection (Burke *et al.*, 1997). Therefore, its colocalisation with SGII, as a marker of the total cellular compliment of granules, should mirror that of the tagged Rab proteins if these preferentially label newly synthesised granules. Furthermore, colocalisation between each construct and SGII should improve over time,


A
B
C
ppANF-EGFP
Anti-SGII
Overlay
D
E
F
EGFP-Rab3A
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Overlay
18h
G
H
I
EGFP-Rab27A
Anti-SGII
Overlay
J
K
L
ppANF-EGFP
Anti-SGII
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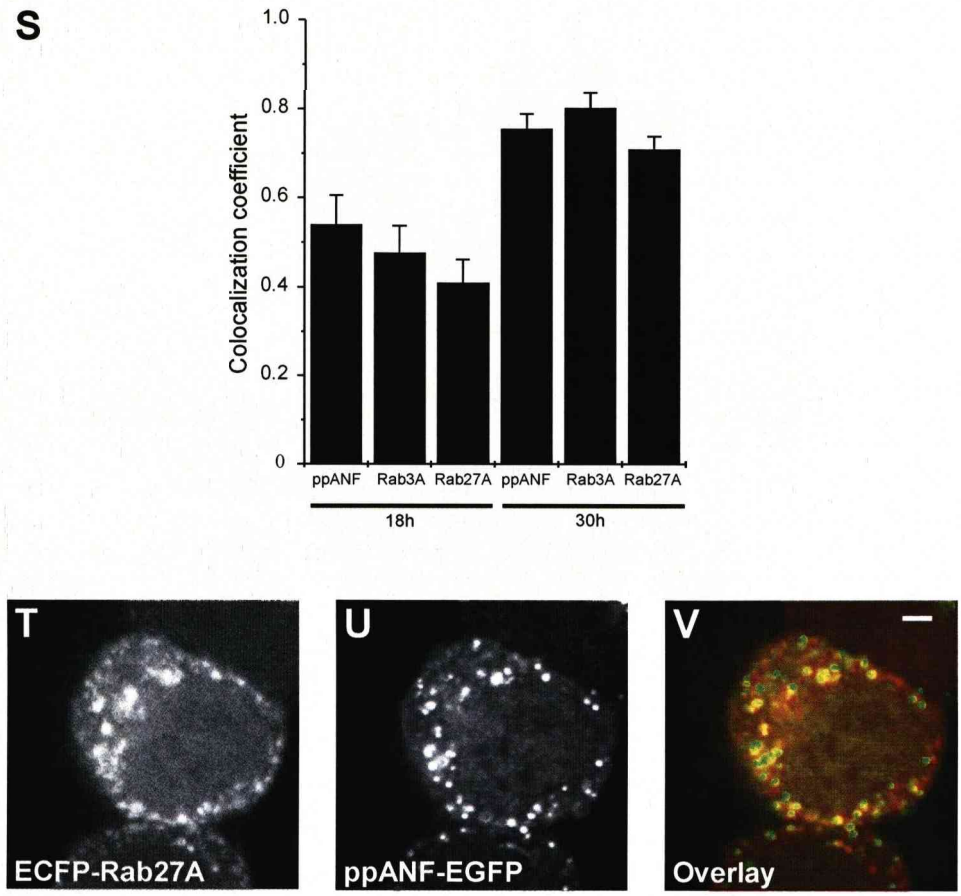


Figure 3.3. Improved colocalisation between ppANF-EGFP/EGFP-Rab3A/EGFP-Rab27A and secretory granules over time post transfection. PC12 cells were transfected with ppANF-EGFP (A-C, J-L), EGFP-Rab3A (D-F, M-O) or EGFP-Rab27A (G-I, P-R) and fixed 18 hours or 30 hours post transfection as indicated. After fixation, cells were immunostained with anti-secretogranin II (anti-SGII). (S) Quantification of the increased colocalisation of EGFP and SGII seen over this time. (T-V) PC12 cells cotransfected with ECFP-Rab27A and ppANF-EGFP, and fixed 18 hours after transfection. Each overlay image on the right of the figure is a composite of green from the left image and red from the centre image. Areas of overlap appear in yellow. Quantitative data shown for the correlation coefficient as mean ± s.e.m. (n=5) Bars, 4μm.

as granules synthesised post-transfection make up a greater proportion of the total. Cells were transfected with ppANF-EGFP, EGFP-Rab3A or EGFP-Rab27A, fixed at 18 hours and 30 hours post-transfection, probed with anti-SGII, and compared (Fig.3.3, A-R). Quantitative analysis showed that at each timepoint, colocalisation between each construct and SGII was strikingly similar, while it was partial 18 hours after transfection, and significantly more extensive after 30 hours (Fig 1.3, S). Since there was

such similarity between ppANF-EGFP and tagged Rab protein fluorescence, the extent of their colocalisation was determined. When ppANF-EGFP was cotransfected with ECFP-Rab27A (Fig.3.3, T-V), the proteins were found to colocalise strongly.

The above data provide strong evidence that the tagged Rab proteins are preferentially recruited to relatively newly synthesised secretory granules, and so it was decided to more precisely determine the time course of their recruitment. To do this, a blockade of vesicle budding at the TGN produced when cells are incubated at 20°C was utilised. This technique has been used previously to follow immature granules in PC12 cells (Rudolf *et al.*, 2001). Following culture for 18 hours at 20°C and fixation, cells expressing ppANF-EGFP showed accumulated fluorescence in a perinuclear region, as expected if it was trapped in the TGN (Fig.3.4, A). In contrast, EGFP-Rab3A and EGFP-Rab27A showed a more diffuse cytosolic distribution (Fig.3.4, B-C). Although punctate structures were visible in many cells, indicating that a proportion of the Rabs can associate with pre-existing granules, these results do indicate a deficit in the recruitment of Rabs to existing granules in the absence of normal granule biogenesis. Switching of cells to 37°C for 30 minutes prior to fixation altered the localisation of all three of the constructs (Fig.3.4, D-F). This relief of the blockade of vesicle budding led to the appearance of labeled punctate structures throughout the cells, indicating that ppANF-EGFP-containing granule biogenesis was initiated rapidly, and that the Rabs were able to interact with granules early in this process.

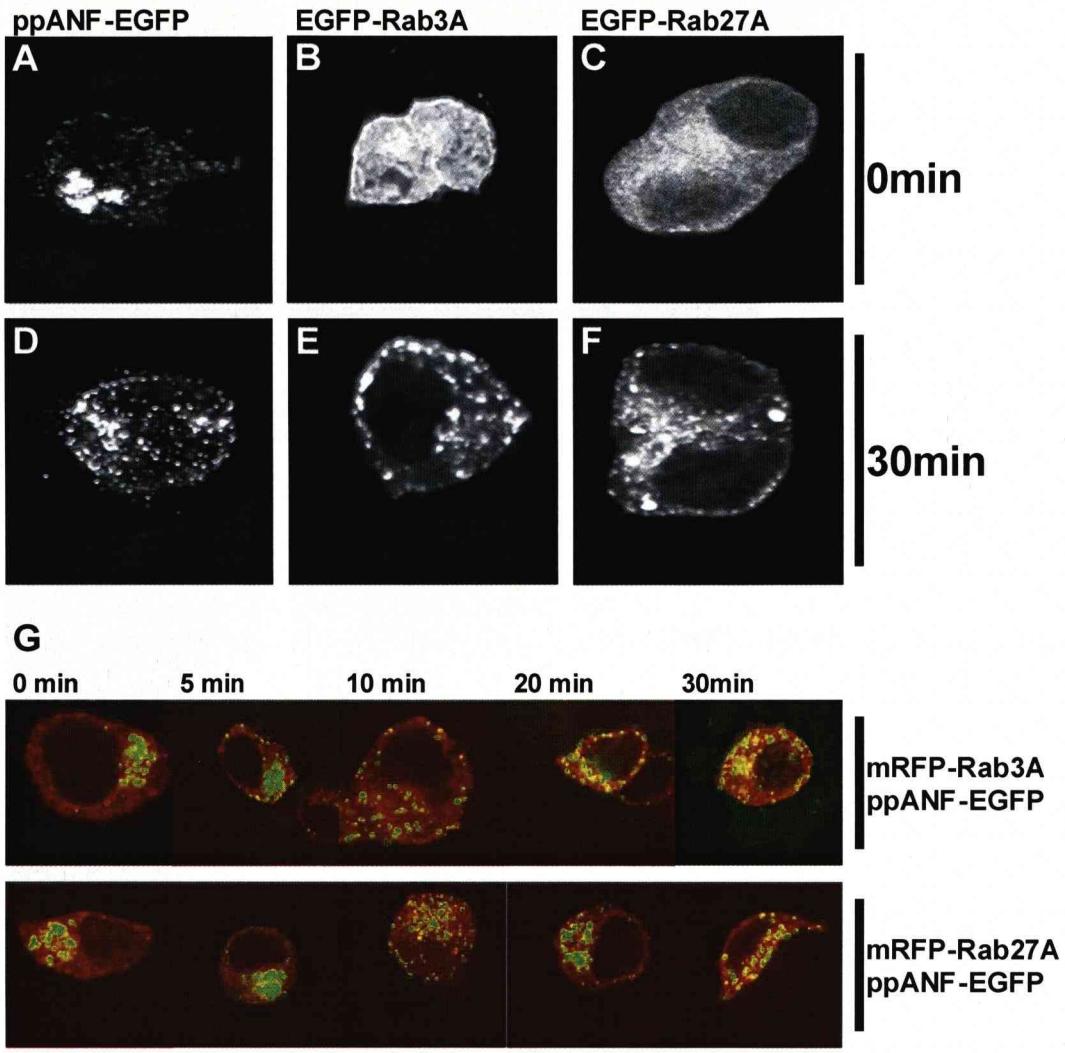


Figure 3.4. Appearance of Rab3A and Rab27A on immature secretory granules during granule biogenesis and maturation. (A-F) PC12 cells were transfected with ppANF-EGFP, EGFP-Rab3A or EGFP-Rab27A and cultured for 18 hours at 20°C to block granule budding at the TGN and then fixed (time 0) or incubated for 30 minutes at 37°C to allow budding of immature granules. (G) PC12 cells were co-transfected with ppANF-EGFP and mRFP-Rab3A or mRFP-Rab27A, cultured for 18 hours at 20°C and then fixed at the indicated times after incubation at 37°C. The images show overlays of ppANF-EGFP fluorescence in green and mRFP fluorescence in red.

Other studies in PC12 cells have shown that immature secretory granules move within seconds to the cell periphery following their budding from the TGN, and after a further period of tens of minutes become immobilised in the cell cortex (Rudolf *et al.*, 2001). During maturation secretory granules increase in size and specific components are removed from them with a half-time of around 45 minutes (Tooze *et al.*, 1991; Dittie *et al.*, 1997).

Since it was found that Rab recruitment was effected within 30 minutes following the lifting of the 20°C temperature-block, it is likely that these proteins were recruited to immature secretory granules at some stage during their maturation.

To follow the time course of their association with immature granules, cells were transfected to coexpress ppANF-EGFP and Rab3A or Rab27A tagged with monomeric red fluorescent protein (Fig.3.4, G). Immature secretory granules labeled with ppANF-EGFP were visible throughout the cells after 5-10 minutes following incubation at 37°C. However, colocalisation between the ppANF-EGFP and mRFP-Rab3A or mRFP-Rab27A was not evident until the cells had been incubated at 37°C for 20 minutes or more, indicating a lag period between release of immature secretory granules from the TGN and the association of the Rab proteins of up to 20 minutes.

3.2.2 Rab activity and exocytosis

Following the characterisation of Rab protein recruitment to secretory granules, the functional effects of this recruitment were explored. Rab3A overexpression is reported to be inhibitory (Holz *et al.*, 1994; Regazzi *et al.*, 1996; Johannes *et al.*, 1998), though it may enhance spontaneous exocytosis (Schluter *et al.*, 2002; Sons and Plomp, 2006). Rab27A overexpression is variously reported to inhibit or enhance exocytosis (Yi *et al.*, 2002; Desnos *et al.*, 2003; Menasche *et al.*, 2003; Johnson *et al.*, 2005). In PC12 cells, several groups have reported that overexpression of

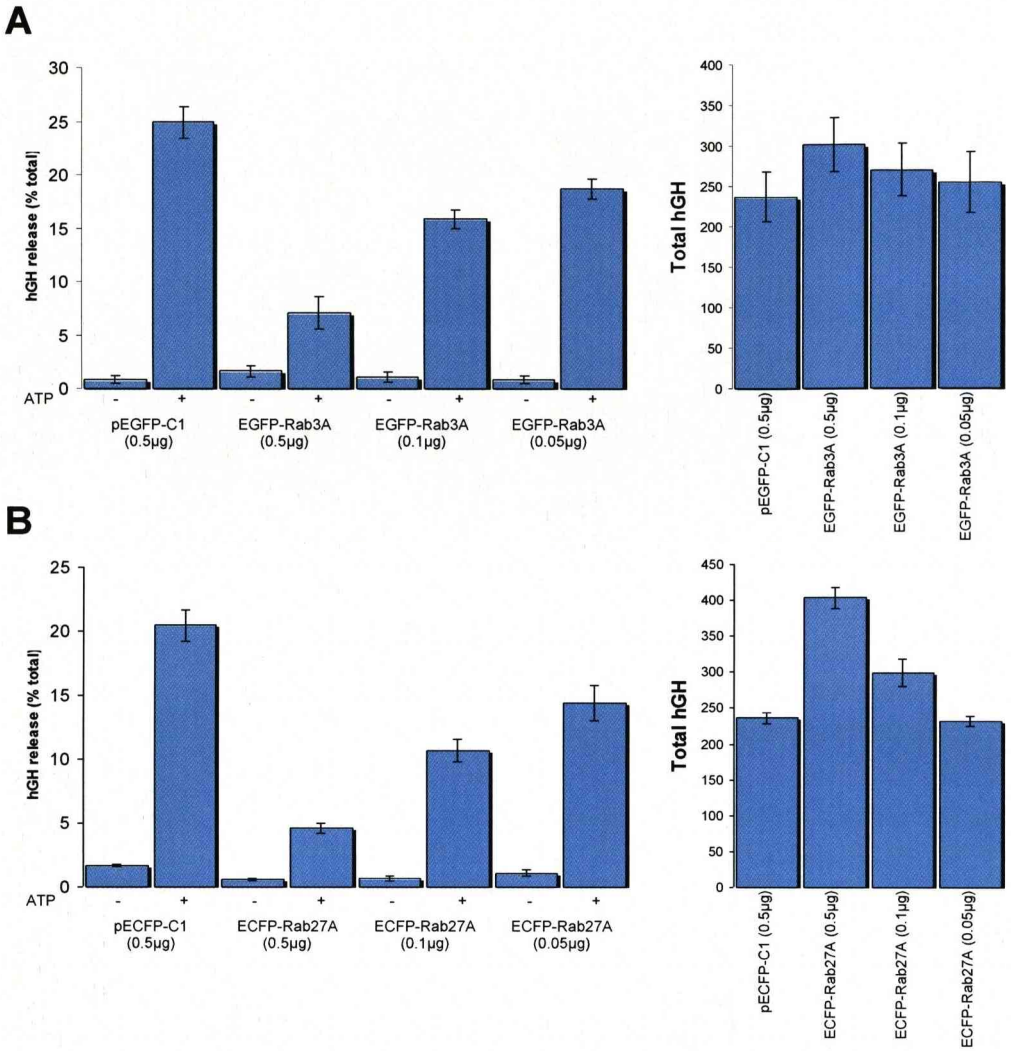


Figure 3.5. Exogenous Rab proteins affect secretion of exogenous hGH. Secretion of exogenous human growth hormone (hGH) from cells cotransfected with varying levels of plasmids encoding EGFP-Rab3A (A) or ECFP-Rab27 (B) 48 hours before stimulation was assayed. Stimulated cells were exposed to 300 µM ATP, hGH release over 15 minutes was assayed and expressed as a percentage of total hGH (shown to the right of the figure). Data are expressed as mean ± s.e.m. (n=6)

either Rab3A or Rab27A inhibits secretion (Schluter *et al.*, 2002; Desnos *et al.*, 2003). It was therefore necessary to determine whether the constructs already used in this study produced the same functional effects. It was possible to employ hGH cotransfection assays for this determination because of the very high cotransfection efficiency of PC12 cells (Graham *et al.*, 2000). Cells were cotransfected with a range of concentrations of

fluorescently tagged Rab expression vectors and an hGH expression vector. pEGFP-C1 or pECFP-C1 vector was added to transfection mixes where applicable so that each mix contained the same final DNA concentration. 48 hours post-transfection, cells were stimulated with 300μM ATP, secreted hGH was assayed, normalised with respect to total hGH, and compared with hGH secreted from unstimulated cells. Transfection with as little as 50ng EGFP-Rab3A or ECFP-Rab27A vector DNA was found to significantly inhibit hGH secretion, while where higher levels of vector were used, a greater degree of inhibition was seen (Fig.3.5, A-B).

Following synthesis, Rab3A and Rab27A are subjected to C-terminal geranylgeranylation, a post-translational modification essential for their membrane-association and function (Zerial and McBride, 2001; Leung *et al.*, 2006). The unprenylated Rab is bound by a Rab escort protein (REP) that mediates its presentation to the alpha and beta subunits of a geranylgeranyl transferase enzyme (GGTase). REP and GGTase proteins are thought to bind preferentially to GDP-bound Rab proteins (Seabra, 1996). REPs are suggested to participate in multiple rounds of Rab geranylgeranylation, because upon completion of each round, these proteins can mediate initial delivery of prenylated Rabs to membranes (Alexandrov *et al.*, 1994). On the surface of membranes, Rab proteins can become GTP-associated, which is a requirement for their interaction with the majority of Rab effectors (Takai *et al.*, 2001). However, while GDP-bound, Rabs are susceptible to extraction back into the cytosol in a

process involving GDP-dissociation inhibitors (GDIs). GDIs sequester GDP-bound Rab protein within the cytosol, while they are also suggested to participate in the correct targeting of specific Rab proteins to specific membrane compartments (Goody *et al.*, 2005; Leung *et al.*, 2006). Given that the above evidence suggests that the association between Rab proteins and secretory granules is intrinsically linked to their nucleotide-binding, it was decided to explore the effects of mutations that affect that binding on the localisation and function of Rab27A.

ECFP-Rab27A point-mutants with reduced intrinsic GTPase activity (Q78L), and defective nucleotide-binding (N133I), were generated by site-directed mutagenesis (SDM). Because of its reduced GTPase activity, the Q78L mutant is thought to be predominantly in a GTP-bound state under physiological conditions (Desnos *et al.*, 2003; Fukuda, 2003b). The N133I mutation is analogous to a mutant of Ras, N116I, in which defective nucleotide binding prevents or attenuates the protein's geranylgeranylation (Huang *et al.*, 2001). When either mutant was expressed in PC12 cells, they showed a largely diffuse cytosolic localisation, markedly different from that of the unmutated ECFP-Rab27A control (Fig.3.6, A-C). Because it is difficult to determine by confocal microscopy whether a fraction of a fluorescent protein is not cytosolic where there is a strong signal from a cytosolic fraction, these data could not distinguish a complete arrest in the targeting of the mutant proteins to secretory granules from a partial reduction in this targeting. Therefore, another series of experiments were carried out in which cells transfected with the mutants were digitonin-

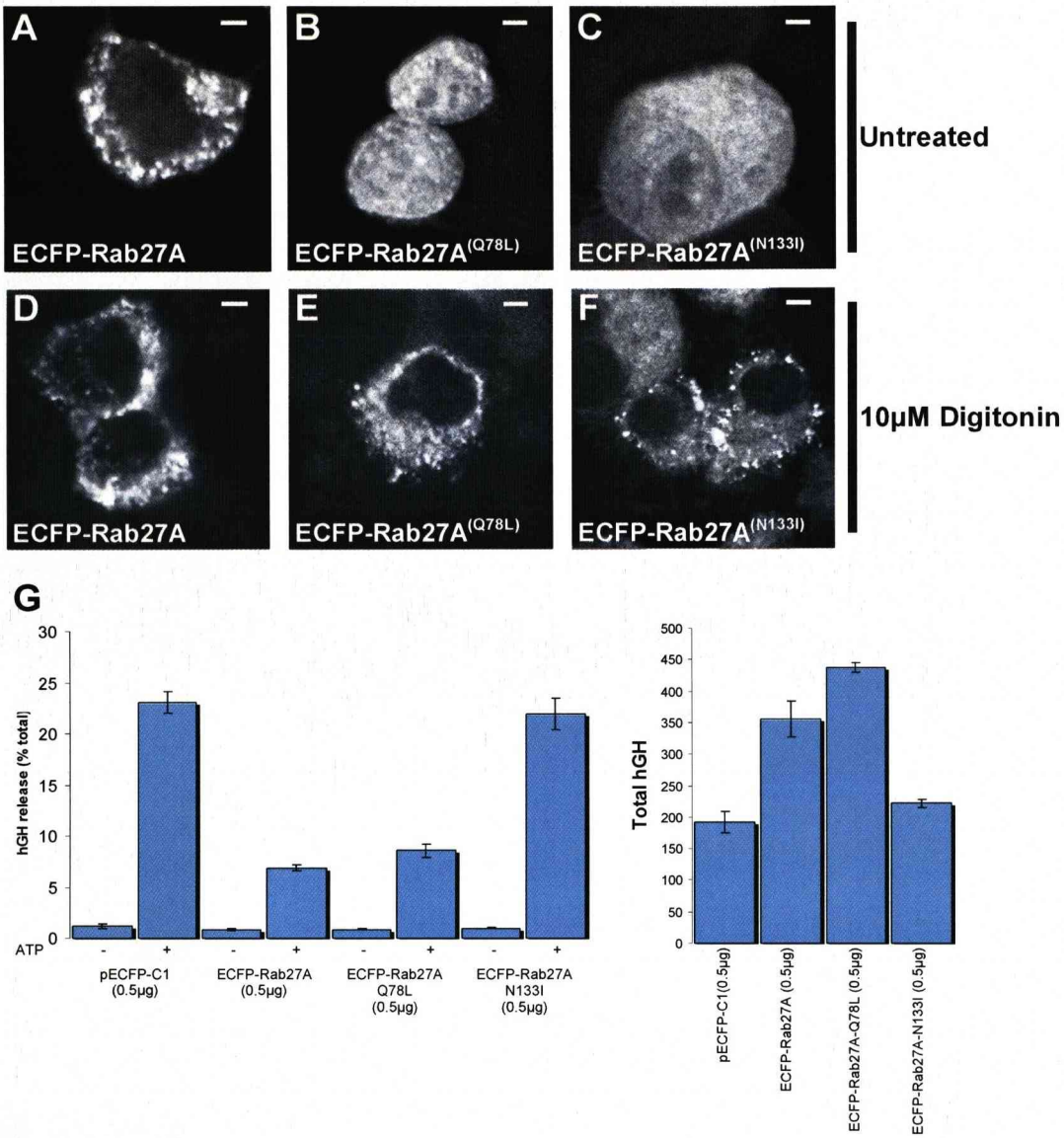


Figure 3.6. Effects of Q78L and N133I mutations on ECFP-Rab27A localisation and function. (A-C) Cells fixed 18 hours after transfection with a plasmid encoding ECFP-Rab27, ECFP-Rab27A (Q78L) or EGFP-Rab27A (N133I) were imaged. (D-F) Cells as above were exposed to a permeablisation buffer containing 10μM digitonin for 15minutes for the removal of free cytosolic protein prior to fixation. Images show the association of each construct with puncta within cells. Bars, 4μm. (G) Secretion of exogenous human growth hormone (hGH) from cells cotransfected with the above plasmids 48 hours before stimulation was assayed. Stimulated cells were exposed to 300 μM ATP, hGH release over 15 minutes was assayed and expressed as a percentage of total hGH (shown to the right of the figure). Data are expressed as mean ± s.e.m. (n=6)

permeablised prior to fixation in order to remove cytosolic protein. As shown in Fig.3.6, D-F, permeablisation revealed an association between the mutants and punctuate structures, likely to be secretory granules, within the cells, suggesting that some targeting to granules still occurred.

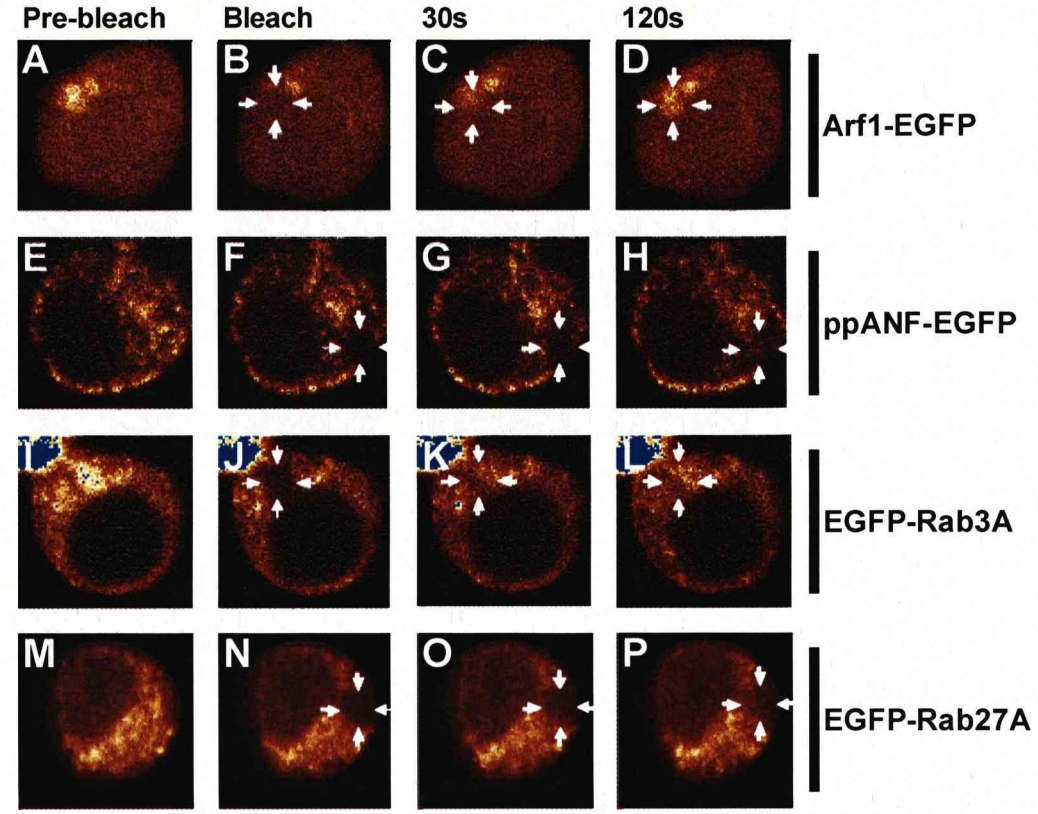
Since the unmutated ECFP-Rab27A construct had been shown to reduce secretion in the hGH cotransfection assay in PC12 cells, the effects of the mutant proteins were determined using the same system (Fig.3.6, G). While the N133I mutant was found to have no significant effect on hGH secretion compared to the control, the Q78L mutant reduced secretion to almost the same level as the unmutated Rab, despite its apparently significantly reduced secretory granule binding capability.

3.2.3 Rab3A but not Rab27A is dynamically associated with secretory granules in PC12 cells

Recruitment of Rab3A and Rab27A to immature secretory granules is consistent with the suggestion made for synaptic vesicles that once the proteins associate with these vesicles, they are only released during or following exocytosis (Fischer von Mollard *et al.*, 1991; Star *et al.*, 2005). To directly address this possibility, it was decided to use fluorescence recovery after photobleaching (FRAP) experiments. In a FRAP experiment, the fluorescent protein within a small region of interest (ROI) of a cell is bleached using a high-intensity laser, and the recovery of fluorescence within this area is subsequently monitored (Lippincott-Schwartz and Patterson, 2003). The recorded fluorescence recovery reflects the replacement of bleached protein in the ROI with unbleached protein from outside this region. To test whether the protocol that was to be used could successfully resolve exchange of fluorescent protein between cytosol and membrane in PC12 cells, bleaching experiments were carried out on

ARF1-EGFP-transfected cells. ARF1 is a small GTPase shown to cycle rapidly between cytosolic and Golgi-associated states in other cell types (Vasudevan *et al.*, 1998; Presley *et al.*, 2002). Expressed ARF-1-EGFP was partially cytosolic but also showed a clear localisation to a perinuclear region presumed to be the Golgi complex in PC12 cells (Fig.3.7, A). Following the bleaching of an area of the Golgi complex, fluorescence recovery of this ARF-1-EGFP occurred with a $t_{1/2}$ of 44 seconds and was almost complete (fractional recovery of 87.5%) within the recorded timeframe of the experiments (Fig.3.7, A-D, Q). This behaviour was similar to that described for ARF1 previously, and confirms the applicability of the FRAP protocol in PC12 cells.

Following bleaching of fluorescently tagged EGFP-Rab3A or EGFP-Rab27A, fluorescence recovery in the ROI could be attributed to three sources: the diffusion of unbleached cytosolic protein, exchange between bleached and unbleached protein on granule membranes, and the movement from other parts of the cell of granules carrying unbleached protein. Compared with the other components of recovery, diffusion of unbleached protein into the ROI was likely to be rapid (~1 second), whereas recovery as a result of granule movement was initially an unknown quantity. For the latter aspect, the recovery following bleaching of ppANF-EGFP was assessed (Fig.3.7, E-H, Q). Since this protein is contained within granules, its recovery profile would be solely due to the effects of granule movement, and provides a reference for comparison with the other data. ppANF-EGFP showed only a slow and partial


Pre-bleach
Bleach
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A
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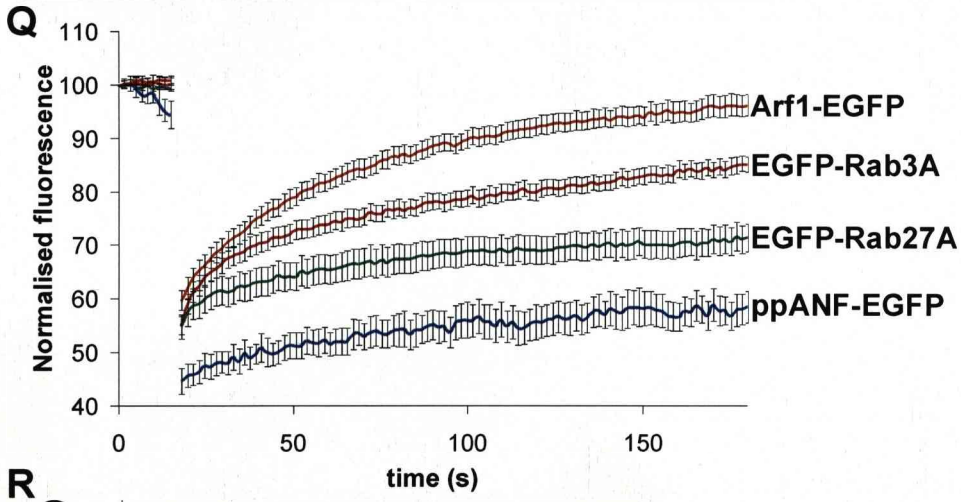
EGFP-Rab27A


Q
Normalised fluorescence
110
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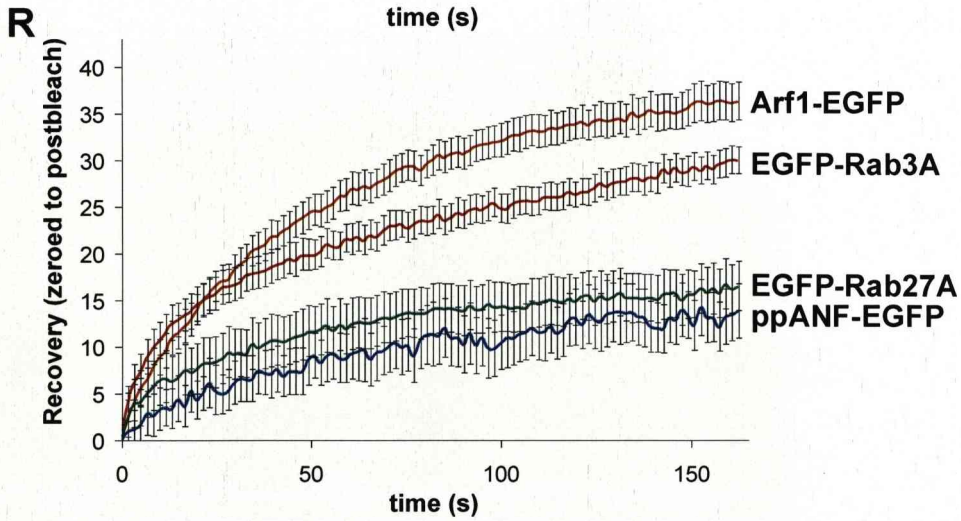
time (s)
Arf1-EGFP
EGFP-Rab3A
EGFP-Rab27A
ppANF-EGFP


R
Recovery (zeroed to postbleach)
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Arf1-EGFP
EGFP-Rab3A
EGFP-Rab27A
ppANF-EGFP

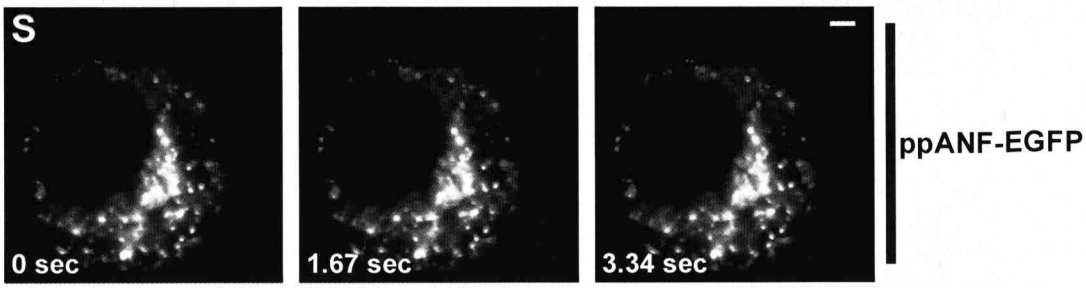


Figure 3.7. Dynamics of EGFP-Rab3A and EGFP-Rab27A on secretory granules in PC12 cells. (A-P) PC12 cells were transfected to express the indicated EGFP-tagged protein and imaged 18 hours following transfection. Regions of interest (ROIs) in each cell were bleached with high intensity laser (arrows) and the fluorescence recovery in these areas was recorded over time. (Q) Plot of fluorescence recovery data corrected for general photobleaching and combined after initial fluorescence for each cell was normalised to 100. (R) Plot of fluorescence recovery as above but with first post-bleaching data point normalised to 0. (S) Three consecutive frames from imaging of a ppANF-EGFP-transfected cell. Data shown are mean ±s.e.m. of 14 ARF1-EGFP, 13 ppANF-EGFP, 21 EGFP-Rab27A and 21 EGFP-Rab3A expressing cells. Bar, 4μm.

fluorescence recovery (fractional recovery of 23.7%) in the bleached region, with a $t_{1/2}$ of 58 seconds. These findings were consistent with limited secretory granule mobility under resting conditions (Burke *et al.*, 1997), a suggestion confirmed here by direct imaging of live cells expressing the construct (Fig.3.7, S).

FRAP experiments were conducted on EGFP-Rab3A and EGFP-Rab27A-transfected PC12 cells 18 hours following their transfection. Only cells expressing relatively low levels of the exogenous protein were selected. For Rab27A (Fig.3.7, M-P, Q), as expected, it was found that proportionate fluorescence recovery at ~1.6 seconds post bleaching (i.e. the first post-bleaching data point) was greater than seen in the ppANF-EGFP-transfected cells, consistent with a greater cytosolic component of recovery. However, when the data were normalised with respect to this initial data point, the recovery profiles of these proteins were not significantly different, suggesting that the minimal EGFP-Rab27A recovery

seen after photobleaching was largely or entirely due to granule movement rather than exchange of Rab27A on the granules (Fig.3.7, R). In the case of EGFP-Rab3A (fractional recovery 66.6%), the recovery profile (Fig.3.7, I-L, Q-R) was strikingly different to that of EGFP-Rab27A (fractional recovery 37.8%). In fact, the recovery of EGFP-Rab3A was more similar to that of the ARF1-EGFP, with extensive, although not complete recovery with a $t_{1/2}$ of 32 seconds. These data suggest that like ARF1 but unlike Rab27A, Rab3A cycles continuously between the cytosol and membranes, and that in PC12 cells at least, this recycling is not exclusively coupled to the exocytotic event and so may be important prior to fusion.

Earlier in this chapter, experiments were described in which exogenous EGFP-Rab3A was shown to associate with a greater proportion of secretory granules at 30 hours post-transfection than at 18 hours post-transfection. In the light of the findings described above, which suggest that the Rab protein associates with granules in a dynamic manner, it is possible to rule out the suggestion that this was due to progressive saturation of Rab3A binding-sites on more recently synthesised granules. This leaves two explanations open. The first is that Rab3A on older granules is unable to cycle between the granules and cytosol. The second is that the capacity of old granules for recruitment of Rab3A is lower than that of newer granules. To test the possibility of a difference in cycling we carried out FRAP experiments on older EGFP-Rab3A-labeled granules in cells at 30 and 54 hours after transfection and compared these to granules in cells at 18 hours post transfection. EGFP-Rab3A recovered with similar

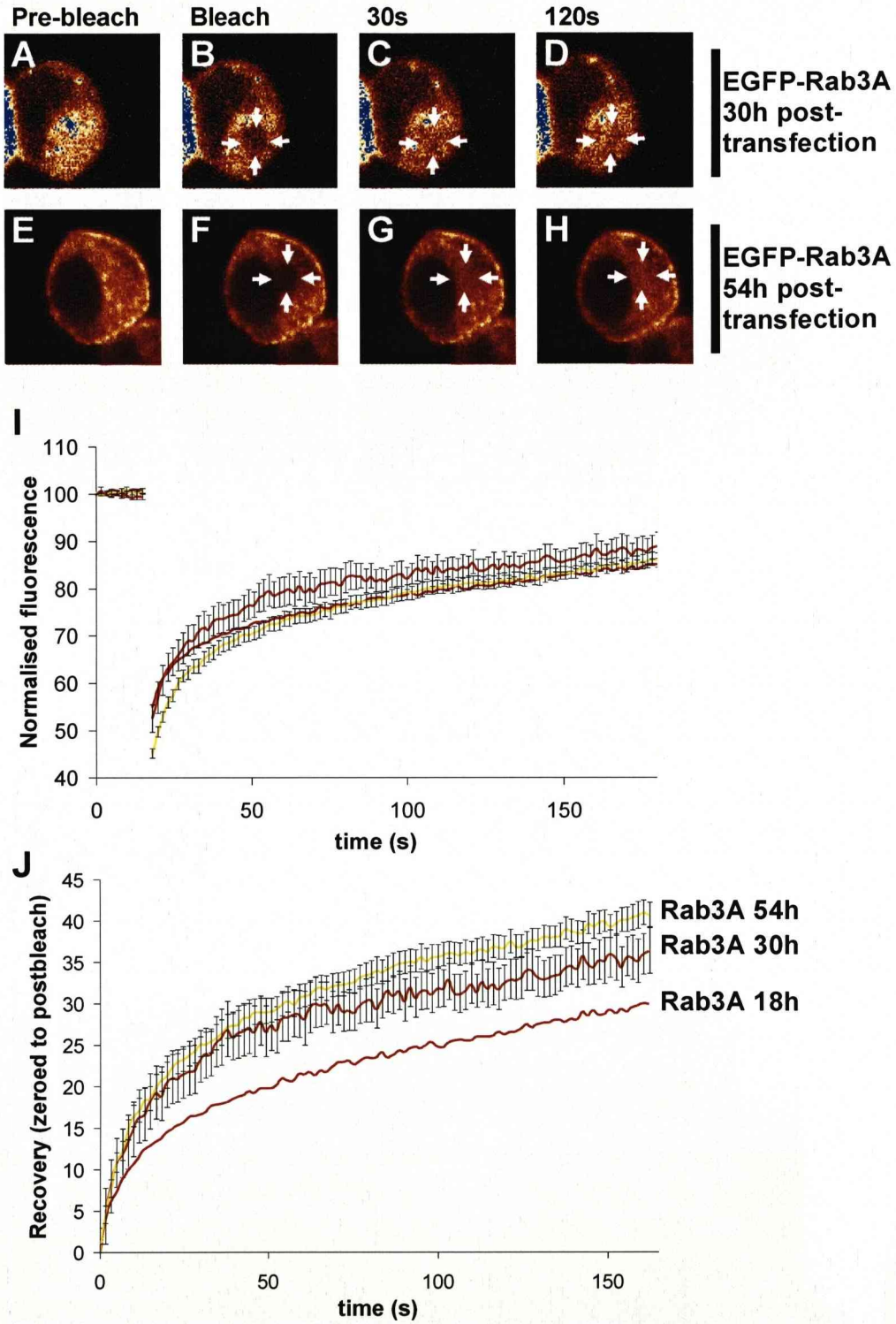


Figure 3.8. Dynamics of EGFP-Rab3A with increasing time post-transfection. (A-H) PC12 cells were transfected to express EGFP-Rab3A and imaged 30 or 54 hours following transfection as indicated. Regions of interest (ROIs) in each cell were bleached with high intensity laser (arrows) and the fluorescence recovery in these areas was recorded over time. (I) Plot of fluorescence recovery data corrected for general photobleaching and combined after initial fluorescence for each cell was normalised to 100. (J) Plot of fluorescence recovery as above but with first post-bleaching data point normalised to 0. Data shown are mean ±s.e.m. of 10 cells at 30 hours and 27 cells at 54 hours.

kinetics onto granules in cells at 18, 30 and 54 hours but with a progressively higher fractional recovery for the older granules (Fig.3.8, A-J). This data supports the second explanation since it indicates that Rab3A can cycle on and off both the newer and the older granules, but may cycle off older granules more rapidly.

Recent work has suggested that Rab protein extraction from membranes involves the heat-shock protein Hsp90 (Sakisaka *et al.*, 2002; Chen and Balch, 2006). More specifically, it was found that extraction of Rab3A from synaptosome membranes was regulated by a chaperone complex containing GDIα, Hsp90, Hsc70 and CSP, and that inhibition of Hsp90 inhibited Ca^{2+}-triggered exocytosis in this system (Sakisaka *et al.*, 2002). While the authors suggest that Hsp90-dependent Rab3A extraction is coupled to exocytosis, it was possible that the continuous cycling of EGFP-Rab3A between membrane and cytosol that we observed was also mediated via this complex. To investigate this possibility, parallel bleaching experiments were carried out on control EGFP-Rab3A-transfected cells and cells incubated with 10 μM geldanamycin for 1 hour before bleaching. Geldanamycin is a potent and specific inhibitor of Hsp90, and was used here at the concentration found to virtually abolish extraction of Rab3A from synaptosomal membranes following stimulation with Ca^{2+} (Sakisaka *et al.*, 2002). The recovery profile for EGFP-Rab3A following photobleaching in control cells was closely similar to that in the earlier experiments (compare Fig.3.7, Q-R and Fig.3.9, A). It was found that recovery profiles for control and treated cells were virtually superimposable

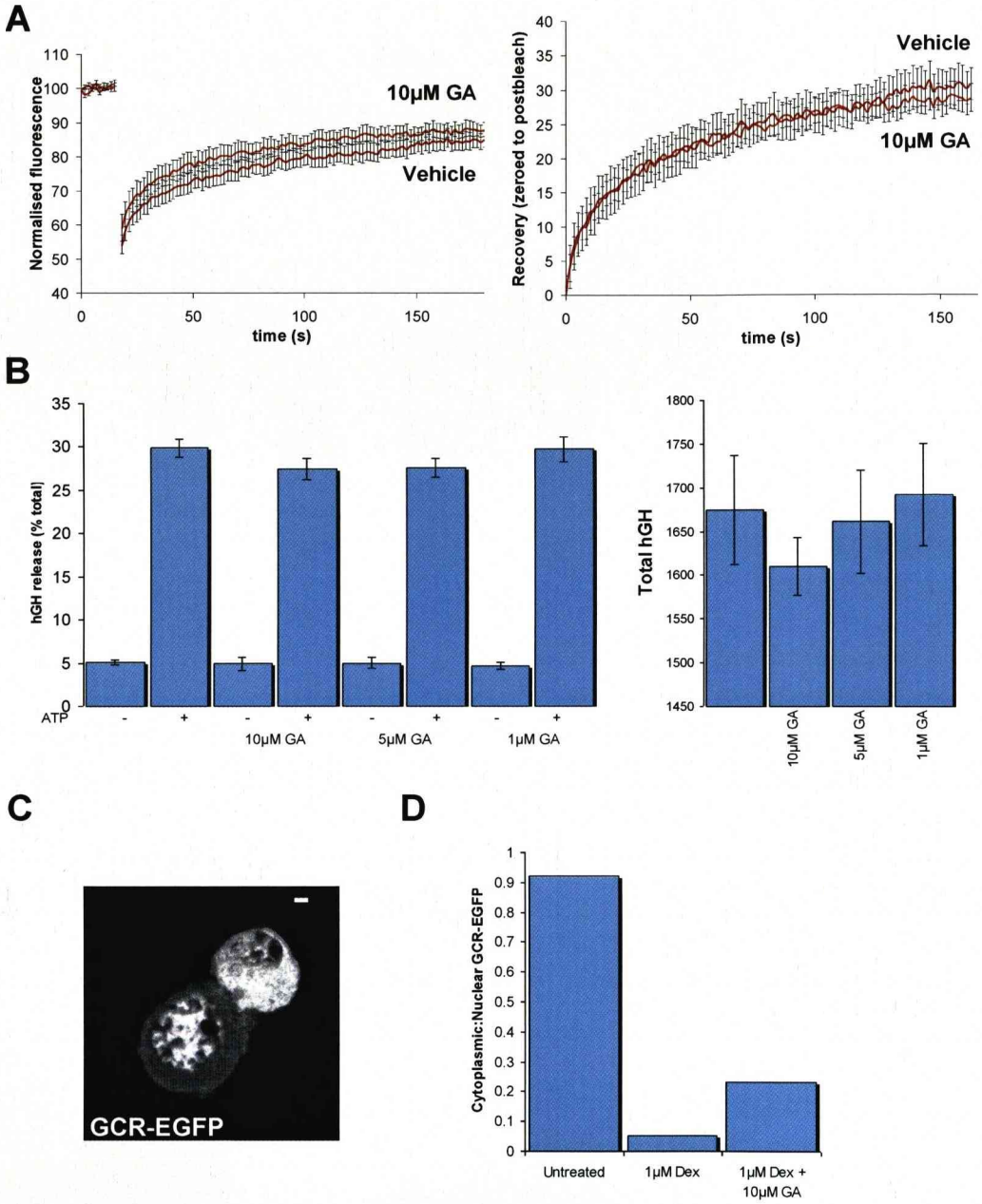


Figure 3.9. Inhibition of HSP90 does not affect EGFP-Rab3A dynamics or exocytosis in PC12 cells. (A) PC12 cells were transfected to express EGFP-Rab3A, and imaged 18 hours following transfection following a 1 hour treatment with vehicle or 10μM geldanamycin (GA). Regions of interest (ROIs) in each cell were bleached with high intensity laser and the fluorescence recovery in these areas was recorded over time. (left) Plot of fluorescence recovery data corrected for general photobleaching and combined after initial fluorescence for each cell was normalised to 100. (right) Plot of fluorescence recovery as above but with first post-bleaching data point normalised to 0. Data shown are mean ±s.e.m. of 11 EGFP-Rab3A expressing cells for each condition. (B) Secretion of exogenous human growth hormone (hGH) was assayed. 18 hours following transfection, cells were treated with GA for 1 hour, then exposed to 300 μM ATP. hGH release over 15 minutes was assayed and expressed as a percentage of total hGH (shown to the right of the figure). Data are expressed as mean ± s.e.m. (n=6). (C) Representative image showing cytosolic or nuclear glucocorticoid receptor (GCR-EGFP). (D) Effect of GA on GCR-EGFP translocation to the nucleus. Treated cells were exposed to 1μM dexamethasone on ice for 1 hour, or 1μM dexamethasone on ice for 1 hour, with the addition of 10μM GA for the final 30 minutes. [contd. on next page].

Figure 3.9 contd. Following a 20-minute incubation at 37°C and fixation, 100 cells were scored for each condition according to the cytosolic or nuclear localisation of the GCR-EGFP. Data are expressed as cytoplasmic:nuclear ratios and are representative of two independent experiments. Bar, 2μm.

(Fig.3.9, A), with $t_{1/2}$ of 29 and 28.6 seconds for control and geldanamycin-treated cells, respectively. This suggests that the resting EGFP-Rab3A cycling observed here occurs independently of Hsp90.

As it had been reported that geldanamycin blocks neurotransmitter release from synaptosomes, its effects on secretion from PC12 cells were assayed here. ATP-stimulated release of exogenously expressed human growth hormone (hGH) from PC12 cells pre-incubated for 1 hour prior to stimulation with varying concentrations of the inhibitor was measured (Fig.3.9, B). The release of hGH was assayed to allow examination of release from newly synthesised granules in transfected cells (i.e. those that exogenous Rabs become associated with). As shown, geldanamycin had no significant effect on hGH release within the range of concentrations used, suggesting that secretory granule exocytosis occurs in an Hsp90-independent manner in this cell type.

It was possible that the negative results described above were caused because the geldanamycin used was inactive, or because PC12 cells were in some way resistant to its effects. To rule out these possibilities, the effects of the inhibitor on the well-characterised Hsp90-dependent nuclear translocation of glucocorticoid receptor (GCR) were determined. It has been found that 10μM geldanamycin can block dexamethasone induced

nuclear translocation of GCR-EGFP in NIH-3T3 cells (Galigniana *et al.*, 1998), and so this protocol was applied to PC12 cells. GCR-EGFP-transfected cells were serum starved, chilled on ice, exposed to 1 μM dexamethasone, and then either vehicle or 10μM geldanamycin. Following a brief period of incubation at 37°C and fixation, 100 cells per treatment expressing moderate amounts of this protein were scored according to its localisation. In individual cells in the cultures, the GCR-EGFP was either predominantly in the cytosol or nucleus (Fig. 1.9, C). As expected, the presence of geldanamycin increased the proportion of cells in which the GCR-EGFP was cytosolic, indicating that it was indeed active in PC12 cells (Fig.3.9, D).

It has previously been reported that activation of exocytosis leads to Rab3A extraction from synaptic membranes (Fischer von Mollard *et al.*, 1991; Star *et al.*, 2005). Indeed, Star et al. have directly imaged the reversible dispersal of EGFP-Rab3A away from presynaptic terminals and into neighbouring axonal regions during periods of synaptic activity. It was unknown, however, whether dissociation and dispersal occurs for Rabs located on dense core secretory granules. In addition, experiments on synaptic Rabs requires use of either biochemical approaches or imaging of whole synaptic terminals whereas study of PC12 cells allows imaging of events on single secretory granules in live cells. Therefore, it was decided to image EGFP-Rab3A and EGFP-Rab27A-transfected cells during stimulation with ATP to raise intracellular calcium and activate exocytosis in PC12 cells. As it had previously been shown in hGH cotransfection

assays that the constructs used in this study are capable of inhibiting exocytosis (Fig.3.5), cells were transfected with only 0.1μg of each construct; a level at which inhibition was in each case found to be less than half maximal. Further, imaging was carried out only 18 hours post-transfection so that expression levels of the Rabs would be less than in the 48-hour hGH experiments, and only those cells expressing low levels of fluorescence were monitored.

Images were taken using settings to image within a thick confocal section to ensure that granules were not lost because of movement out of the confocal section and cells were rejected if any focus drift as a result of cell movement was detected. Initially, ppANF-EGFP-transfected cells were observed to see if the cells underwent exocytosis in response to 300 μM ATP. As noted previously, there is little movement or loss of granules from the imaged field under resting conditions. During stimulation, ppANF-labeled granules in the cell periphery but not those in the cell interior were seen to disappear abruptly often between successive images (Fig.3.10, A), indicating exocytosis of these granules. This can also be seen in the example shown in Fig.3.10, B in which images before (green) and after (red) stimulation have been overlaid so that granules that remained appear yellow. A green 'corona' of released granules can be seen in the ppANF-EGFP-transfected cell. The number of granules lost per cell was variable but on average close to 20% of labeled granules disappeared. By contrast, when EGFP-Rab3A or EGFP-Rab27A-transfected cells were imaged during ATP stimulation, although some short-range granule movement

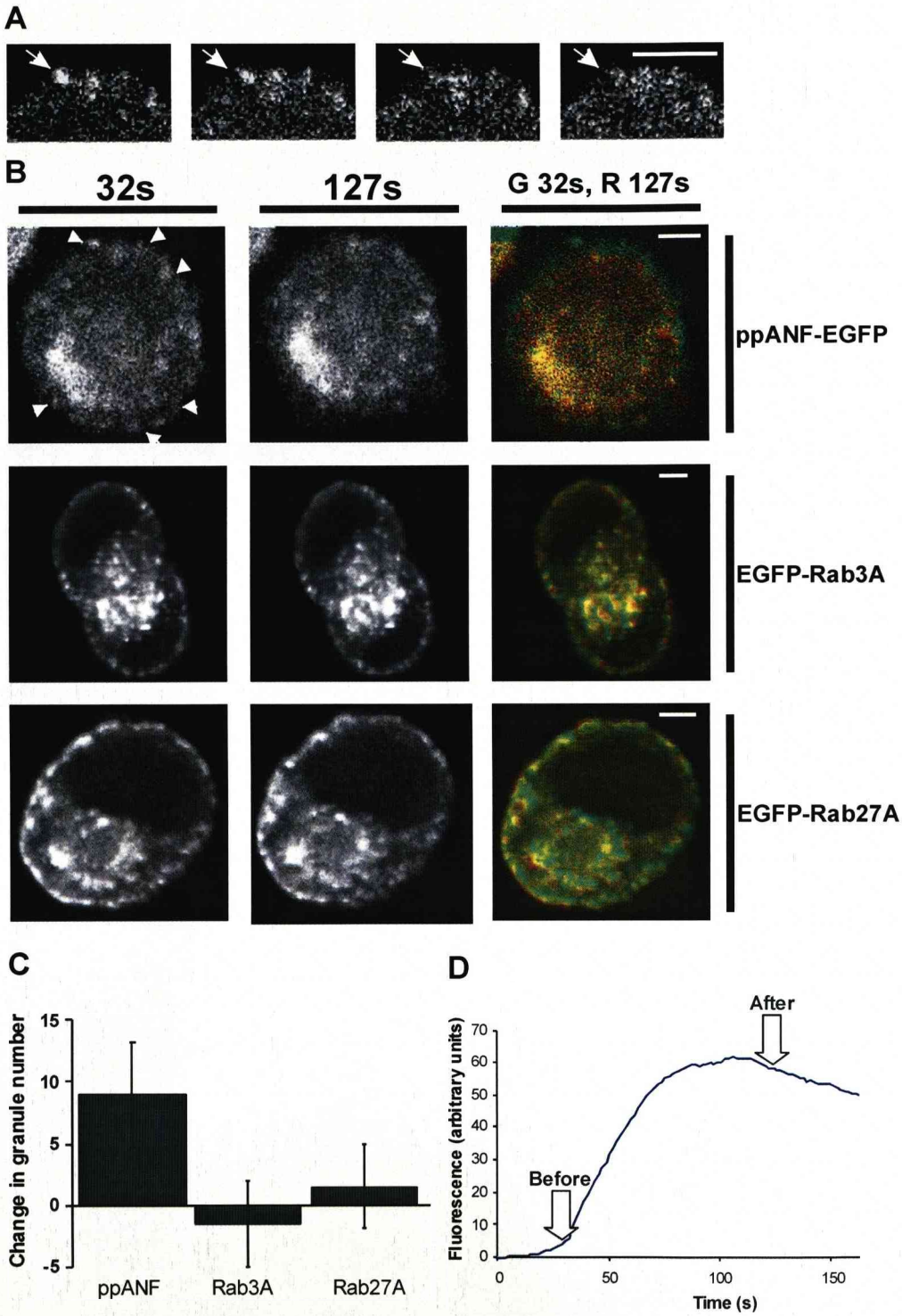


Figure 3.10. Effect of activation of exocytosis on secretory granules labelled with EGFP-ppANF, EGFP-Rab3A or EGFP-Rab27A in live cells. Cells were transfected to express EGFP-ppANF, EGFP-Rab3A or EGFP-Rab27A and imaged 18 hours after transfection. Cells showing low levels of fusion protein expression were selected for observation and stimulated by perfusion with 300 µM ATP. (A) Disappearance of a ppANP-EGFP-labelled granule during stimulation. The images shown are sequential frames in which the sudden disappearance of a granule (arrow) can be observed. (B) Effect of stimulation with 300 µM ATP on secretory granules labelled with EGFP-ppANF, EGFP-Rab3A or EGFP-Rab27A as indicated. Images were taken at 32 seconds and 127 seconds into the series. [contd. on next page]

Figure 3.10 contd. Overlay images are shown with *t*=32 seconds in green and *t*=127 seconds in red so that granules that disappear between the images appear green. Granules that disappeared in the ppANF-EGFP cell are indicated with arrowheads. (C) The number of granules that disappeared between the before and after images was identified by the comparison of images at 19 and 76 seconds, quantified and shown as mean ± s.e.m. for 8 cells expressing each construct. (D) Example of the time course of Ca^{2+} changes from monitoring of X-Rhod fluorescence following ATP stimulation (*n*=11 cells).

was noted, there was no overall loss of fluorescent granules at the cell periphery or in the cell interior (Fig.3.10, C). These results suggest that Rab3A and Rab27A do not immediately disperse from sites of exocytosis at the plasma membrane in PC12 cells.

As an additional control for the above experiments, cells were loaded with the intracellular Ca^{2+} indicator X-Rhod to confirm that they responded to ATP stimulation. An increase in Ca^{2+} concentration was found to occur in all cells analysed and the mean change in X-Rhod fluorescence over the time course of experiments is shown in Fig.3.10, D.

3.3 Discussion

Much of the work described in this chapter was carried out on cells transfected with N-terminally tagged chimeras between fluorescent proteins and either Rab3A or Rab27A. It is therefore important to consider whether tagging and overexpression might have affected normal Rab protein distribution and function before discussing any findings in this context. Overexpression of fluorescently tagged protein can be associated with mislocalisation and aggregation (Shaner *et al.*, 2005). However, several lines of evidence suggest that this did not occur in the experiments described. Both the increased colocalisation between EGFP-Rab3A or EGFP-Rab27A and SGII with time post-transfection (Fig.3.3, A-R), and the strong colocalisation between ECFP-Rab27A and ppANF-EGFP (Fig.3.3, T-V) point to the correct targeting of the chimeras to secretory granules. Severe disruption of this targeting in the ECFP-Rab27A Q78L and N133I point mutants (Fig.3.6, B-C), are almost certainly a result of their specific effects on nucleotide binding, and dynamic exchange of EGFP-Rab3A between granule membranes and cytosol revealed by FRAP (Fig.3.7) does not support the notion that the proteins aggregate. Finally, like their endogenous counterparts (Schluter *et al.*, 2002; Desnos *et al.*, 2003), both EGFP-Rab3A and EGFP-Rab27A were shown to inhibit secretion in hGH cotransfection assays (Fig.3.5), indicating that these proteins are unlikely to be functionally compromised, and EGFP-Rab27A has previously been shown to functionally replace wild-type Rab27A in a mouse 'knock in' model (Tolmachova *et al.*, 2004).

If it can be concluded that the behaviour of the tagged Rab proteins is representative of that of the untagged proteins, it appears very likely that Rabs 3A and 27A are preferentially recruited to newly synthesised granules: the initially partial colocalisation of the exogenous Rabs with SGII (Fig.3.3, D-I); their stronger colocalisation over time post-transfection (Fig.3.3, M-R); the colocalisation between ECFP-Rab27A and EGFP-Rab3A and ppANF-EGFP (Fig.3.1, D-F, Fig.3.3, T-V); and the reduced recruitment of the mRFP-tagged Rabs to punctae in the absence of immature secretory granules under temperature-block (Fig.3.4, B-C) all suggest this. One explanation of this finding would be a model in which granules possess saturable binding sites for Rabs and in which endogenous Rab3A and Rab27A block the association between exogenous Rabs and these sites by occupying them. This would be consistent with reports that Rab3A-binding to synaptic vesicles is saturable, and dependent on a proteinaceous receptor (Chou and Jahn, 2000). The overall picture must however, be more complex, as FRAP experiments in this study demonstrate a more dynamic interaction between EGFP-Rab3A and granules than could be supported in this model.

The data from FRAP analysis showed that Rab3A, but not Rab27A can cycle rapidly between the granule membrane and the cytosol (Fig.3.7, Q). The reproducibility of the FRAP technique is demonstrated by the virtual superimposability of recovery profiles from two independent series of experiments carried out in the presence or absence of geldanamycin

(Fig.3.9, A). While previous studies on Rab3A have only identified neuronal Rab3A dispersal coupled to exocytosis (Fischer von Mollard *et al.*, 1991; Sakisaka *et al.*, 2002; Star *et al.*, 2005), they have not addressed the possibility that cycles of granule/vesicle association and dissociation occur under resting conditions: because they compared membrane and cytosolic levels of Rab3A by Western blotting or changes in fusion protein distribution, it would be impossible to identify a system of exchange at the point of equilibrium. Further, it is possible that the biochemical procedures used could have led to the rapid loss of cycling Rab3A from vesicles, or a rapid loss of Rab3A cycling capability, due to the dilution of cytosol through suspension in a large volume of buffer (Fischer von Mollard *et al.*, 1991), or digitonin-permeablisation (Sakisaka *et al.*, 2002). Indeed, other small GTPases, including Rab5 and Rab7 can exchange between membrane and cytosol (Vasudevan *et al.*, 1998; Mochizuki *et al.*, 2001; Presley *et al.*, 2002). FRAP experiments on EGFP-Rab5 exogenously expressed in CHO cells showed that the bleached protein on phagosomes is rapidly replaced with a $t_{1/2}$ of ~5 seconds (Vieira *et al.*, 2003). EGFP-Rab7 expressed in Mel JuSo cells is replaced on endosomes and lysosomes with a $t_{1/2}$ of ~52 seconds, while 31% of the protein was found to be in an immobile fraction (Jordens *et al.*, 2001). In this context, it may be the finding that Rab27A does not cycle once it becomes granule-associated that is surprising.

How can the preferential recruitment of Rab3A to newly synthesised granules be reconciled with its rapid cycling between granule membrane and cytosol? It may be targeted to saturable binding sites on newly

synthesised granules and initially exchanged between the granule membrane and the cytosol, but over time become progressively more unexchangable, thus blocking any further binding of the cycling pool. Under this model, fluorescence recovery of EGFP-Rab3A would be expected to be incomplete. The EGFP-Rab3A fluorescence recovery profile presented here does not reach a point of maximal recovery, and so it is unclear whether eventual recovery is complete or incomplete. However, EGFP-Rab27A (Fig.3.7, Q-R) and other characterised Rab proteins (Jordens *et al.*, 2001) do show such partial recovery. Furthermore, progressive unexchangability of Rab3A would be consistent with the suggested behaviour of Rab5 and Rab9 (Zerial and McBride, 2001; Ganley *et al.*, 2004; Grosshans *et al.*, 2006). It is suggested that these Rabs can become segregated to stable multimolecular 'Rab domains', and that these domains are resistant to disassembly because of positive feedback activation via RabGEF.

The above model could explain the observed difference between EGFP-Rab3A and EGFP-Rab27A dynamics in that it would suggest that Rab27A becomes unexchangable much more readily than Rab3A. However, the model would also predict that EGFP-Rab3A recovery after bleaching should become progressively more partial with time post-transfection, as older granules carrying more stably associated protein make up an increasingly greater component or the protein that is bleached. This effect was not seen in FRAP experiments conducted in this study. In fact, in experiments carried out 30 hours and 54 hours – as opposed to 18 hours -

post transfection, proportionate fluorescence recovery was unchanged or slightly increased (Fig.3.8, I), and the rate of recovery was slightly more rapid (Fig.3.8, J). These results should be treated with a degree of caution since multiple relevant factors could change with time. For example, the proportion of tightly granule-associated Rab might increase with time, but so might the proportion of cytosolic Rab. Nevertheless, they might lead to an alternative model in which the Rab3A binding capacity of older granules is lower than that of newer granules. Under this model, the observed change in EGFP-Rab3A dynamics with time could be explained if a small fraction of the total cellular Rab exchanging between cytosol and older granules is removed from them, and therefore replaced, more rapidly.

On consideration, the above two models are not mutually exclusive. As granules age, a fraction of the cycling Rab protein may become unexchangable, while the capacity of the granule to bind additional Rab for any length of time may be gradually lost. This 'combined' model would be consistent with data presented here, as well as findings showing that GDP-bound but not GTP-bound Rab proteins are susceptible to GDI-mediated extraction from membranes (Chou and Jahn, 2000), and those showing that RabGEFs required to catalyse exchange of GDP for GTP on membrane-bound Rabs and 'activate' them, may become incorporated into stable multimolecular 'Rab domains' (Zerial and McBride, 2001). Together, these data suggest a system in which, as secretory granules age, 'Rab3A domains' may form and sequester Rab3GEF, thus lowering the capacity of the remainder of the granule membrane to 'activate' additional Rab. This

would increase that Rab's susceptibility to GDI-extraction and thereby limit the amount of time for which this additional protein remains membrane-associated. See diagram below.

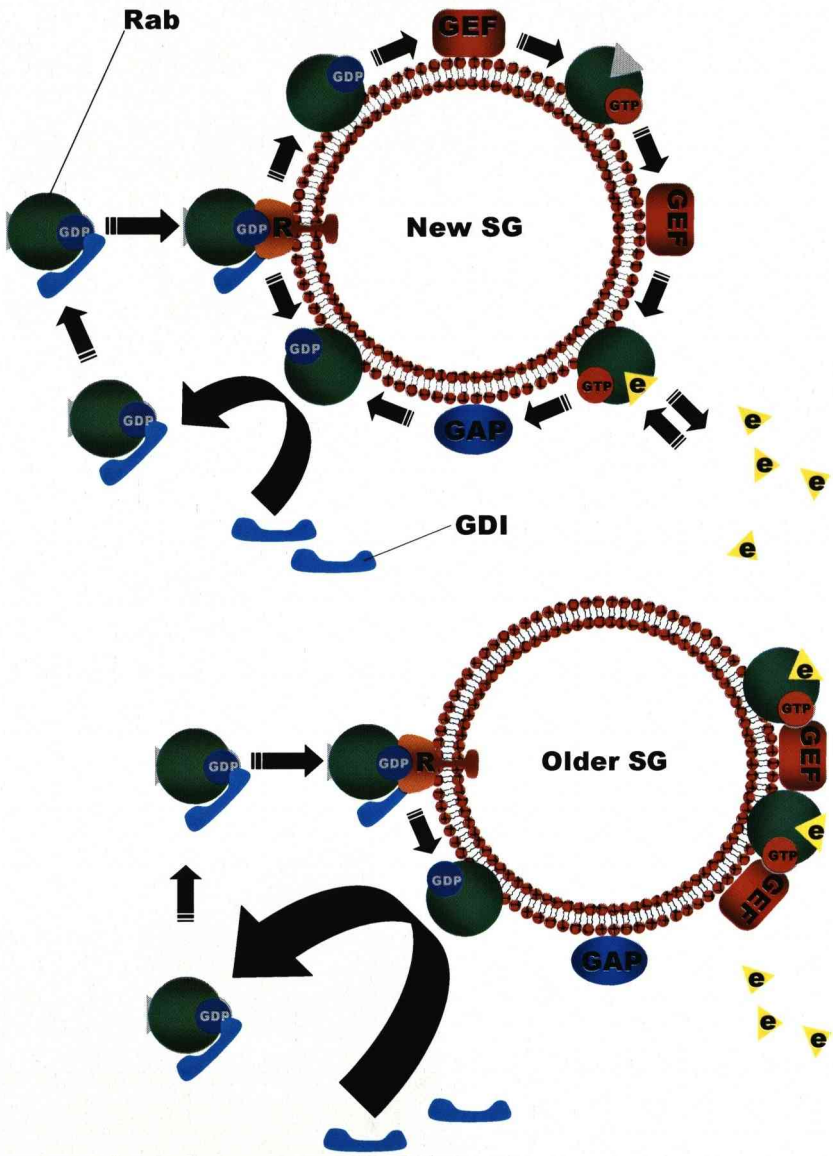


Figure 3.11. Model for accelerated Rab3A extraction from secretory granules with increasing granule age. On new granules, GDP-bound Rab protein complexed with GDP dissociation inhibitor (GDI) is displaced by a granular receptor (R), with GDI displacement factor (GDF) activity. The GDP-Rab may be activated through interaction with a GDP-GTP exchange factor (GEF), or extracted from the membrane by a GDI. GTP-bound Rab protein may bind effectors, or be inactivated through interaction with a GTPase activating protein (GAP). On older granules, GTP-bound Rab protein may sequester GEF protein in a stable 'Rab domain', so that additional GDP-Rab protein delivered to the granule membrane is less likely to be activated, and more likely to undergo GDI-dependent extraction. Figs. 3.1-4 suggest preferential recruitment of Rab3A to newly synthesised granules, Figs. 3.7-8 suggest increasingly dynamically associated component to granular Rab3A, Fig. 3.10 suggests stably associated component to granular Rab3A.

What is the mechanistic basis for the observed difference between Rab27A and Rab3A dynamics? According to the above model, one suggestion would be that Rab27A could become more rapidly unexchangable as a result of its incorporation into 'Rab domains'. More simply though, it might become unexchangable as a result of a larger proportion of the protein maintaining a prolonged GTP-bound state on secretory granules. In agreement with this suggestion, the membrane-associated protein is predominantly GTP-bound in unstimulated platelets (Kondo *et al.*, 2006), while membrane-associated Rab3A in PC12 cells is only ~50% GTP-bound (Burstein *et al.*, 1993). A factor in producing this difference may be the intrinsic GTP hydrolysis rate of Rab27A, which has been found to be much lower than that of Rab3A and Rab5A in vitro ($0.0058min^{-1}$ compared to $0.082\ min^{-1}$ and $0.164\ min^{-1}$ respectively)(Larijani *et al.*, 2003). However, this low intrinsic GTPase activity cannot entirely account for the relative unexchangability of Rab27A, since Rab7, which has a comparable GTP hydrolysis rate of $0.0023min^{-1}$ (Shapiro *et al.*, 1993), retains the capacity to cycle between membrane and cytosol (Jordens *et al.*, 2001).

Other factors that might impact on the membrane-bound state of Rab27A include the relative accessibility and activity of regulatory GEF and GAP proteins on the granule membrane, the effects of any interaction between the Rab protein and its effectors, the susceptibility of the GDP-bound Rab to extraction from the membrane, and, as previously stated, the incorporation of the GTP-bound Rab into 'Rab domains'. One recent

finding that might speculatively provide a mechanism for the relative stability of Rab27A membrane-association comes from a structural study on the related protein Rab27B (Chavas *et al.*, 2007). When crystallised in its GDP-bound form, this protein was found to form a 'swapped dimer' in which switch I and II regions are suggested to be stabilised (Chavas *et al.*, 2007). Although this dimer was not identified in vitro in solution, it is plausible that its formation on membranes in vivo might shield Rab27B from GDIs. Rab9A and Rab11A are also shown to dimerise when crystallised, and their dimerisation is suggested to confer protection from membrane-extraction (Pasqualato *et al.*, 2004; Wittmann and Rudolph, 2004). Consistent with the suggestion that Rab27A may be protected in a similar way, GDP-Rab27A is found to remain associated with secretory granule membranes following stimulation of platelets (Kondo *et al.*, 2006).

In this study, the 'constitutively active' Q78L mutant of ECFP-Rab27A, which is likely to possess reduced GTPase activity, was found to be largely cytosolic (Fig.3.6, B). In a context in which GTP-bound Rab proteins are retained on membranes (see above), this finding is surprising. However, several explanations are possible. Nascent Rab27A is suggested to exist in a largely GTP-bound state, and this may contribute to its less efficient REP/GGTase-mediated geranylgeranylation when compared to other Rab proteins since both REPs and GGTases bind GDP-bound Rabs preferentially (Seabra *et al.*, 1995; Seabra, 1996; Larijani *et al.*, 2003). If the Q78L mutation increased the proportion of GTP-bound Rab27A still further, this might affect its post-translational modification and thereby its

ability to associate with granule membranes. Another explanation of the mutant's localisation might be that the mutation affects its regulation. The Rab3A Q81L mutant - which is analogous to the Rab27 Q78L mutant – is present, in PC12 cells, in a GTP-bound form, only to a similar extent to the wild-type protein despite its reduced GTPase activity (Brondyk *et al.*, 1993). It was discovered that this was because the mutant was sensitive to Rab3GAP, while insensitive to Rab3GEF. Speculatively, if the Q78L mutation were to have a similar effect on Rab27A, the resulting change in the protein's nucleotide-binding dynamics might reduce its activation on granules and/or enhance its extraction. Further, a reduction in its sensitivity to Rab27GEF might be expected to selectively disrupt any 'Rab domains', as a stable GEF-Rab association, and 'positive activation loop', is thought to underlie their formation (Grosshans *et al.*, 2006). On the other hand, should wild-type GDP-Rab27A become resistant to GDI-mediated extraction from membranes in vivo through dimerisation, the Q78L mutant might be more readily extracted if the mutation prevents stable formation of the dimer.

Despite the presence on secretory granules of a proportionately smaller component of the expressed mutant (Fig.3.6, B), ECFP-Rab27A Q78L was found to inhibit secretion in an hGH cotransfection assay to a similar degree to the unmutated protein (Fig.3.6, G). Since that inhibition was found to be concentration dependent (Fig.3.5, B), it is possible that the mutant was simply more highly expressed, although this is unlikely since expression levels of analogous Q81L and wild-type Rab3A in PC12 cells is

found to be indistinguishable by flow cytometry (Holz *et al.*, 1994). It seems more likely that the mutant protein produced a more potent inhibitory effect than the wild type. In the light of possible explanations for the mutant's cytosolic localisation (see above), this finding would be open to conflicting interpretations. If a smaller proportion of the mutant protein was recruited to granules as a result of reduced post-translational modification, then its inhibitory effect may relate to increased interaction of the minimal amount of successfully modified and 'constitutively active' protein on the granule membrane with its effectors. It might also relate to a 'dominant-negative' effect should unsuccessfully modified, 'constitutively active', protein in the cytosol bind and sequester effectors due to a reduced ability to bind GDIs. If a smaller proportion of the mutant protein was recruited to granules as a result of altered regulation of its nucleotide-binding on the granule membrane, then its inhibitory effect might reflect an increased transience in its interactions with its effectors, perhaps as a result of the disruption of 'Rab domains' or dimers. This would be an interesting possibility, as it would imply that for Rab27A, these domains/dimers act to moderate the protein's inhibitory function.

One identified mechanism of rab3A extraction, is that following stimulation of synaptosomes, and involving ATP hydrolysis via an Hsp90-containing complex (Sakisaka *et al.*, 2002). Hsp90 inhibition by geldanamycin in this system led to reduced Rab3A extraction, and reduced exocytosis. However, it was found here, that the maximal concentration of inhibitor used in that study had no effect on 'resting' Rab3A cycling in PC12 cells as

identified by FRAP, nor did it affect secretion (Fig.3.9). While it is formally possible that a higher concentration of inhibitor may have revealed some involvement of HSP90, it was sufficiently high to inhibit dexamethasone-induced nuclear translocation of GCR-EGFP (Fig.3.9, C-D), and such a concentration may also have produced undesired non-specific effects such as inhibition of kinase-regulated cascades (Richter *et al.*, 2001; Young *et al.*, 2001).

One interpretation of the above data is that the mechanism for membrane-extraction of Rab3A differs between PC12 cells and neurons. However, Chen and Balch (Chen and Balch, 2006) reported that an Hsp90-dependent mechanism of Rab extraction operates on other Rab subtypes and in various cell types, making this interpretation unlikely. Another interpretation is that separate mechanisms exist for Rab extraction during 'resting' cycling and for extraction following fusion between a donor membrane compartment and its target membrane. Under this interpretation the Hsp90-dependent mechanism would only control Rab3A extraction following exocytosis. This may be a plausible suggestion, if it is considered that removal of the Rab during 'resting' cycling may involve the removal of the Rab alone, whereas removal of the Rab at the target membrane may require the active disassembly of Rab-containing complexes. While the mechanism for the 'resting' cycling of Rab3A remains obscure, this suggestion would strengthen the argument that some proportion of granular Rab3A could form a component of a stable structure such as a 'Rab domain'. By analogy, it might also offer some

explanation for the retention of GDP-Rab27A on granules in stimulated platelets (Kondo *et al.*, 2006): the disassembly of stable Rab27A-containing complexes might require more than GTP-hydrolysis .

The lack of a detectable effect of geldanamycin on secretion from PC12 cells (Fig.3.9, B) suggests that HSP90-mediated dissociation of Rab3A and Rab27A from membranes is not an integral aspect of the exocytotic process in this cell type. What is the explanation of this finding given that the same compound potently inhibits exocytosis from synaptosomes (Sakisaka *et al.*, 2002)? One characterised difference between neuronal and neuroendocrine exocytosis, is the rapid recycling and refilling of synaptic vesicles in presynaptic terminals (Fernandez-Alfonso and Ryan, 2006). Is it possible that rapid HSP90-dependent recycling of Rab3A, and the resulting increase in cytosolic GDI-bound GDP-Rab3A enhances its recruitment to these newly recycled vesicles? Neuronal exocytosis has been long suggested to be coupled to rapid Rab3 dispersal (Fischer von Mollard *et al.*, 1991; Sakisaka *et al.*, 2002; Star *et al.*, 2005), and it may be that the rapidity of the process reflects a functionally important specialisation of this cell type rather than a mechanism conserved in all cells. In PC12 cells, dispersal of Rab3A is unlikely to so dramatically affect its cytosolic concentration, and in this study, no evidence was found that granule-associated Rab3A and Rab27A disperse when these cells were stimulated (Fig.3.10). Further, evidence has been provided for very rapid retrieval of secretory granules following exocytosis (Graham *et al.*, 2002; Holroyd *et al.*, 2002), and it is therefore possible that Rab3A and Rab27A

remain in stable complexes in order to be retrieved as part of this divergent process.

It should be noted, that recent work by another group, utilizing total internal reflection microscopy (TIRFM), has identified some dispersal of EGFP-Rab3A following exocytosis of secretory granules from PC12 cells (Lin *et al.*, 2007). In cells expressing the secretory granule content marker neuropeptide Y-EGFP (NPY-EGFP), ~56% of NPY-EGFP-containing secretory granules visible within an evanescent field very close to the cells' plasma membranes were seen to disappear within 20 seconds of stimulation. While a similar decrease in NPY-EGFP fluorescence was seen in control cells and cells cotransfected with Rab3A, dispersal of EGFP-Rab3A on stimulation of EGFP-Rab3A-transfected cells was associated with only ~4% of labeled granules. These data do not necessarily conflict with the data presented and discussed above, if it is concluded that the high resolution of the TIRFM-based assay identified EGFP-Rab3A dispersal from only a small proportion of exocytotic sites.

Recent work by Schluter et al. has improved understanding of the function of Rab3A in synaptic exocytosis and suggested that Rab3A boosts the release probability of a subset of the vesicles of the readily releasable pool (Schluter *et al.*, 2004). This finding presents the question as to how Rab3A, which is present on all vesicles, can regulate the properties of only some. It may be relevant that in chromaffin cells, one factor regulating the release probability of secretory granules is granule age, with newly

synthesised granules reportedly released in preference to older granules (Duncan *et al.*, 2003). The molecular basis for this difference between populations of synaptic vesicles and between old and new granules is unknown. However, since we describe data suggesting that Rab3A dynamics in PC12 cells change as granules age, it is an intriguing possibility that a cycling pool of Rab3A in neurones and in neuroendocrine cells is a functionally important determinant of both phenomena. In the context of the whole cell then, cycles of Rab3A membrane association and dissociation may firstly ensure the correct targeting of the protein, and secondly, functionally segregate target organelles according to their age, expanding the range of exocytotic responses that they can mediate.

Chapter 4:

Rab effectors in neuroendocrine exocytosis

4.1 Introduction

Rab proteins are thought to associate with specific membrane compartments and to regulate the functionally diverse, and functionally discrete, multimolecular systems that control various aspects of membrane trafficking (Zerial and McBride, 2001). A given Rab protein may regulate one or several processes that are important for a specific membrane trafficking step, while the basis for such regulation is thought to be interactions between Rabs proteins and effector proteins (Takai *et al.*, 2001; Fukuda, 2005). It is thought that the multiplicity of functions that some Rab proteins exhibit reflects their interaction with multiple effectors (Fukuda, 2005). For example, it is thought that interactions between Rab3A and Synapsins may be important for coordinating interactions between synaptic vesicles and the synaptic cytoskeleton, while its interactions with Rab-interacting molecule (RIM) proteins may be important for coordinating interactions between vesicles and presynaptic active zones (Giovedi *et al.*, 2004a; Giovedi *et al.*, 2004b; Kaeser and Sudhof, 2005). As well as interacting with different effectors within the same cell, a given Rab protein may interact with different effectors in different cell types reflecting specialisation of a given trafficking step according to cellular needs. For example, in melanocytes, Rab27A links melanosomes to the motor protein Myosin Va via its effector Melanophilin (Wu *et al.*, 2002), while in PC12 cells it links secretory granules to Myosin VIIa via its effector MyRIP (Desnos *et al.*, 2003).

Rab proteins are often required for the recruitment of their effectors to a specific membrane compartment, and are likely to be involved in the spatio-temporal control of their activity. In support of this, it is found that most Rab-effector interactions are regulated by the nucleotide-bound state of the Rab, and that many, but not all effectors, bind exclusively to GTP-bound Rab protein (Fukuda, 2005). However, until now, few studies have focused on the dynamics of Rab effectors involved in regulated secretion. On the basis that experiments conducted earlier in this study had successfully characterised aspects of the recruitment and dynamics of Rab3A and Rab27A in PC12 cells, it was decided to widen the scope of the investigation to include selected effectors of these proteins.

4.2 Results

4.2.1 Localisation of exocytotic Rab effectors

In order to study the recruitment and dynamics of Rab protein effectors that associate with secretory granules, it was decided to apply some of the same techniques previously used in the characterisation of Rab3A and Rab27A. To generate vectors for the exogenous expression of wild type or fluorescently tagged effector proteins in PC12 cells, coding sequences for Rabphilin, Granuphilin and Slp-5 were amplified from a PC12 cell cDNA library. Amplification of these sequences from a cDNA library produced from the PC12 cells used in this laboratory demonstrates the transcription of mRNAs encoding these effector proteins in these cells. The coding sequence for Noc2, another Rab effector, was available following previous work (Haynes *et al.*, 2001), and was kindly provided by L. Haynes. Transcription of Noc2 in PC12 cells has been identified by another group through the use of Northern Blot analysis (Kotake *et al.*, 1997).

Vectors encoding EGFP-tagged Rab effectors were transfected into PC12 cells in order to determine the localisation of these proteins (Fig.4.1). Rabphilin-EGFP, EGFP-Rabphilin, and Noc2-EGFP (Fig.4.1, A-B and G respectively) were found to localise to punctate structures distributed throughout the cytosol and at the periphery of cells. This pattern was like that of EGFP-Rab3A and EGFP-Rab27A described in the previous chapter (Fig.3.3), and was consistent with the reported association of the effectors with secretory granules (Chung *et al.*, 1999; Cheviet *et al.*, 2004a; Fukuda

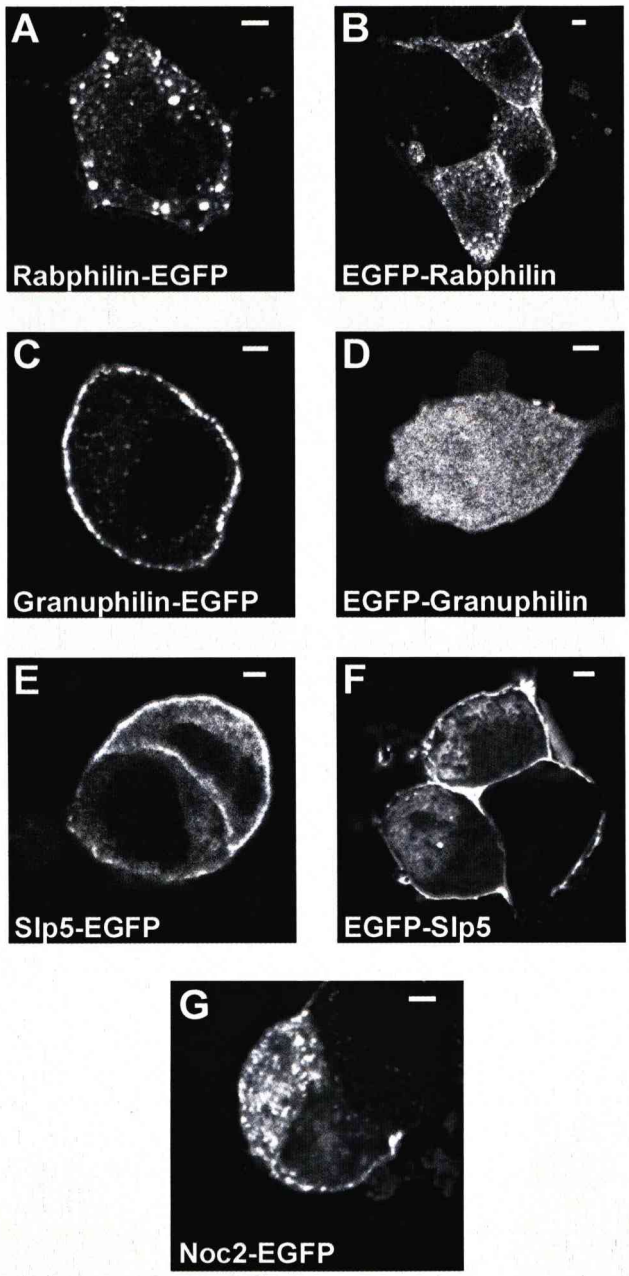
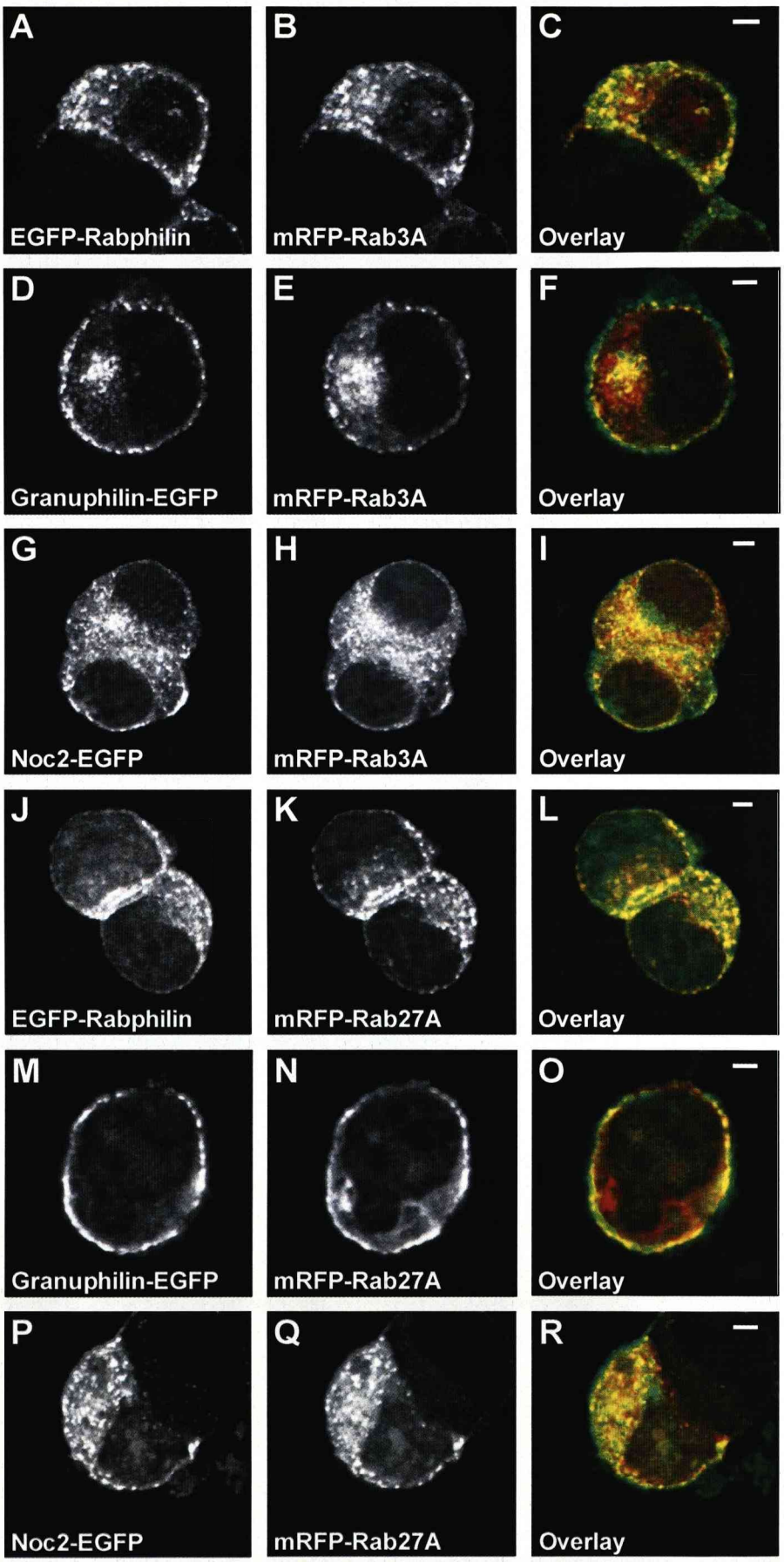


Figure 4.1. Expression of EGFP-tagged exocytotic Rab effectors in PC12 cells. (A-G) Cells were fixed 18 hours following transfection with a plasmid encoding the indicated fusion protein. Bars, 4μm.

et al., 2004). Granuphilin-EGFP was also found associated with punctate structures, although these structures were largely present at the periphery of cells (Fig.4.1, C). This is consistent with findings showing that Granuphilin promotes granule tethering/docking in PC12 cells as gauged by TIRFM (Tsuboi and Fukuda, 2006b), and those showing that

overexpressed HA-tagged Granuphilin, in the pancreatic β-cell-derived Min6 cell line, associates with peripheral granules and promotes granule targeting to the plasma membrane (Torii *et al.*, 2002; Torii *et al.*, 2004; Gomi *et al.*, 2005). While N-terminally tagged mRFP-Granuphilin expressed in PC12 cells in another study appears to be granule-associated (Tsuboi and Fukuda, 2006b), it was found here that N-terminally tagged EGFP-Granuphilin was largely cytosolic (Fig.4.1, D), presumably because the fluorescent tag disrupted the normal function of the protein. Both Slp-5-EGFP and EGFP-Slp-5 were found to show a peripheral localisation, while in this case no punctae were evident. As Slp-5 has previously been reported to bind strongly to Rab27A, and to colocalise with secretory granules (Tsuboi and Fukuda, 2006b), it is likely that the EGFP-tagging of the protein led to its mislocalisation.

To further characterise the localisation of the effector proteins, PC12 cells were cotransfected with EGFP-tagged effector, and either mRFP-Rab3A or mRFP-Rab27A (Fig.4.2). While Rabphilin-EGFP and EGFP-Rabphilin showed similar localisation, N-terminally tagged Rabphilin has been used in previous studies (McKiernan *et al.*, 1996; Tsuboi and Fukuda, 2005; Tsuboi *et al.*, 2007) and so EGFP-Rabphilin was used in these experiments. Granuphilin-EGFP was used in preference to EGFP-Granuphilin since the latter protein appeared to mislocalise. Fluorescently tagged Slp-5 was not used. Both EGFP-Rabphilin (Fig.4.2, A-C, J-L) and Noc2-EGFP (Fig.4.2, G-I, P-R) were each found to colocalise very strongly with both of the mRFP-tagged Rab proteins. This is consistent with the


A
EGFP-Rabphilin
B
mRFP-Rab3A
C
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D
Granuphilin-EGFP
E
mRFP-Rab3A
F
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G
Noc2-EGFP
H
mRFP-Rab3A
I
Overlay
J
EGFP-Rabphilin
K
mRFP-Rab27A
L
Overlay
M
Granuphilin-EGFP
N
mRFP-Rab27A
O
Overlay
P
Noc2-EGFP
Q
mRFP-Rab27A
R
Overlay

Figure 4.2. Colocalisation between EGFP-tagged exocytotic Rab effectors and mRFP-tagged Rab proteins in PC12 cells. Cells were cotransfected with EGFP-Rabphilin (A-C, J-L), Granuphilin-EGFP (D-F, M-O) or Noc2-EGFP (G-I, P-R) and either mRFP-Rab3A (A-I), or mRFP-Rab27A (J-R), and fixed 18 hours following transfection. Each overlay image on the right of the figure is a composite of green from the left image and red from the centre image. Areas of overlap appear in yellow. Bars, 4μm.

suggestion that these effectors localise to secretory granules, while it may also indicate that they, like the tagged Rab proteins, are preferentially recruited to newly synthesised granules. However, it is also possible that since the Rab proteins were overexpressed, effector recruitment to newer granules resulted from an increase in the levels of the Rab proteins on these granules and thereby an increase in the number of effector recruitment sites. Granuphilin-EGFP showed more limited colocalisation with the tagged Rabs, largely confined to perinuclear regions, and to punctae at the periphery of cells (Fig.4.2, D-F, M-O). There was some indication that coexpression of the Granuphilin construct led to the partial redistribution of mRFP-Rab protein-labeled punctae towards a more peripheral localisation (compare Fig.4.2, E with B and H, and N with K and Q). There was also some indication that mRFP-Rab coexpression caused redistribution of some Granuphilin-EGFP to a perinuclear region (compare Fig.4.2, E and N, with Fig.4.1, C). Thus, it appears that Granuphilin-EGFP, and the mRFP-tagged Rab constructs, label overlapping populations of secretory granules, while the Granuphilin may promote targeting of granules to the plasma membrane in PC12 cells as previously reported (Tsuboi and Fukuda, 2006b). This behaviour may reflect the capacity of Granuphilin to bind to Syntaxin and Munc18 proteins (Coppola *et al.*, 2002; Torii *et al.*, 2002; Torii *et al.*, 2004; Fukuda *et al.*, 2005; Tsuboi and Fukuda, 2006b) and/or phosphatidylinositol (4,5)bisphosphate (PIP_2)

(Osborne *et al.*, 2007), each of which are enriched at the plasma membrane.

4.2.2 Rab effector function

The overexpression of Rabphilin, Granuphilin, Slp5 or Noc2 has been previously linked to differing effects on regulated exocytosis. Overexpression of Rabphilin in adrenal chromaffin cells is found to enhance nicotinic AChR agonist-stimulated (DMPP-stimulated) secretion of cotransfected hGH (Chung *et al.*, 1995). Its overexpression in PC12 cells is reported to produce no effect on cotransfected hGH release in response to high-K^+-stimulation (Komuro *et al.*, 1996), although it is also reported to increase high-K^+-stimulated release events associated with cotransfected neuropeptide-Y-Venus (NPY-Venus) (Tsuboi and Fukuda, 2005). In contrast, overexpression of Granuphilin is found to inhibit high-K^+-stimulated secretion of endogenous insulin from Min6 cells (Torii *et al.*, 2002) and high-K^+-stimulated secretion of cotransfected NPY from PC12 cells (Fukuda *et al.*, 2002; Fukuda, 2003b). Slp5 or Noc2 overexpression in PC12 cells was reported to enhance high-K^+-stimulated secretion of cotransfected NPY (Fukuda, 2003b; Fukuda *et al.*, 2004), while Noc2 overexpression was also found to inhibit cotransfected hGH release from permeablised PC12 cells in response to Ca^{2+} (Haynes *et al.*, 2001), and from INS-1E cells in response to multiple secretagogues (Cheviet *et al.*, 2004a).

In the light of the above, sometimes conflicting, data, it was necessary to characterise the effects of effector overexpression on secretion as determined by the assay used in this laboratory. As for analysis of the effects of Rab overexpression (see Chapter 3), PC12 cells were cotransfected with test and hGH-expression vectors, and 48 hours after transfection, hGH secretion in response to 300μM ATP was assayed and compared to that from unstimulated cells. ATP-stimulation produces a secretory response because it activates purinergic receptors leading to calcium influx (Kim and Rabin, 1994). It was used here in preference to high-K^+-stimulation, as it is a more effective secretogouge in PC12 cells. Stimulation through application of Ca^{2+} to permeablised cells was not used because cell permeablisation could lead to the loss of cytosolic Rabs, effectors, or other factors involved in their function.

pcDNA3.1 vectors encoding untagged effector protein were used in each cotransfection experiment in order that the effects of their overexpression could not be attributed to the presence of a fluorescent tag. 0.5μg of each vector was used per transfection, because transfection with this amount of the EGFP-effector-encoding constructs (see previous section) had been found to minimise the number of cells displaying aberrant morphology as a result of excessive expression of exogenous protein. Expression from these constructs, and from the pcDNA3.1-derived constructs, is driven by an identical cytomegalovirus (CMV) promoter. Overexpression of wild type Rabphilin, Granuphilin or Slp5 in these experiments was found to produce little or no effect on ATP-stimulated hGH secretion, although there was

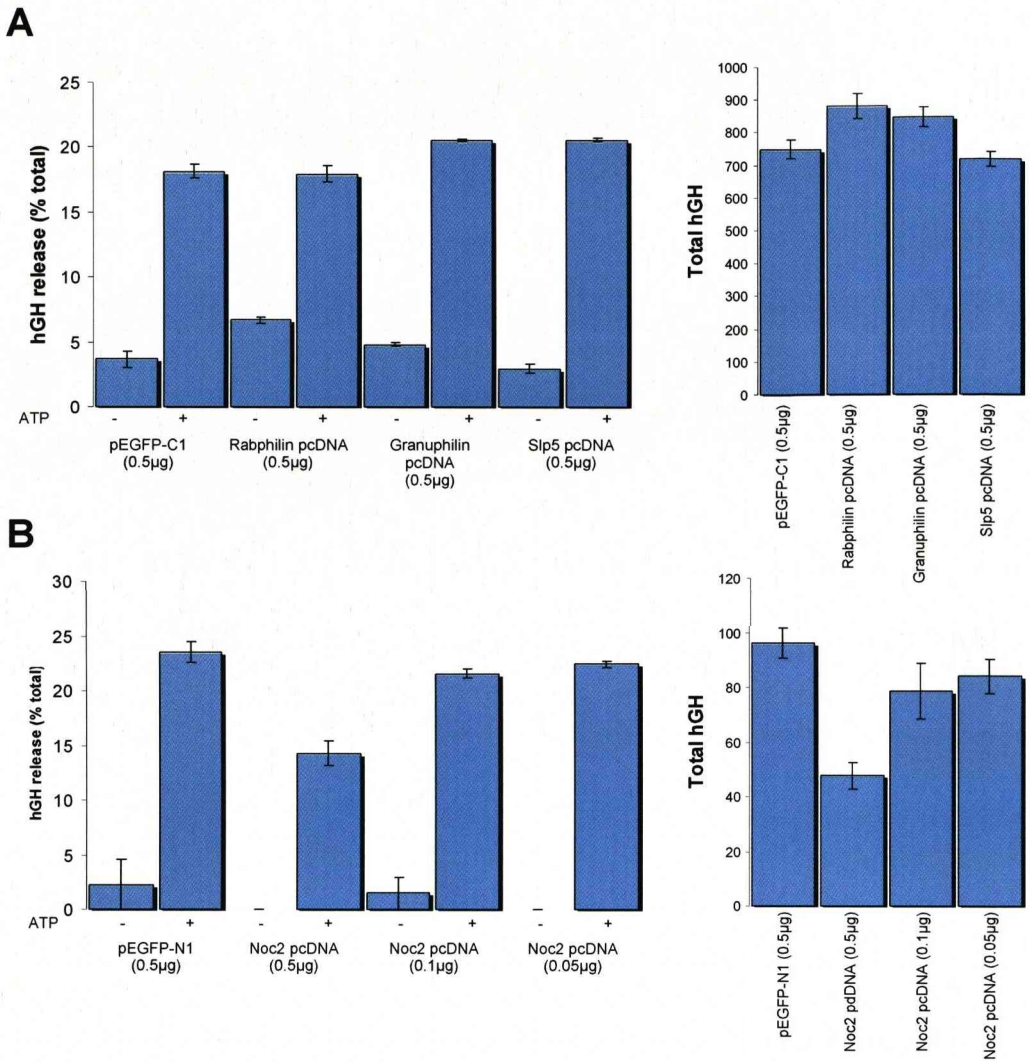

Figure 4.3. Exogenous Rab effectors affect secretion of exogenous hGH. Secretion of exogenous human growth hormone (hGH) from cells cotransfected with plasmids encoding Rabphilin, Granuphilin or Slp5 (A) or varying levels of a plasmid encoding Noc2 (B), 48 hours before stimulation was assayed. Stimulated cells were exposed to 300 µM ATP, hGH release over 15 minutes was assayed and expressed as a percentage of total hGH (shown to the right of the figure). Data are expressed as mean ± s.e.m. (n=6)

some indication that Rabphilin enhanced basal secretion (Fig.4.3, A). In contrast, overexpression of Noc2 (0.5µg/well) produced a marked inhibitory effect (Fig.4.3, B). While it was not demonstrated that each construct was equally well expressed, it appears from these data that Noc2 has a significant role in secretory granule exocytosis as an inhibitory Rab effector.

Because Noc2 overexpression produced a consistent inhibitory effect on secretion, it was possible to determine the functional effects of mutations in the protein that reduce or abolish its interaction with Rab proteins. Previous work on the structure of a Rab3A-Rabphilin complex showed that two adjacent sites on Rabphilin were important for their interaction (Ostermeier and Brunger, 1999) and that a mutation in one of these sites, V61A, could attenuate this interaction in vitro (Joberty, 1999). Later work in this laboratory showed that an analogous, V58A, mutation in Noc2, which shares homology to Rabphilin in this region, could also attenuate an in vitro interaction with Rab3A, while mutation of residues in the area analogous to the second site, by which a SGAWFY structural element was changed to SGAAAA (W154A, F155A, Y156A), could abolish it (Haynes *et al.*, 2001). It was then found that while wild type Noc2 and the Noc2(V58A) mutant interacted comparably with Rab27A, the 'Noc2(AAA)' mutations abolished this interaction as well (Cheviet *et al.*, 2004a).

Before determining the effects of the above mutations on the inhibitory action of Noc2 on secretion, Noc2, Noc2(V58A) and Noc2(AAA) cDNAs were subcloned into pEYFP-N1 expression vectors in order that fluorescence microscopy could be used to identify whether the mutations affected protein localisation. Cells were cotransfected with each of these constructs and ECFP-Rab27A, as a marker for secretory granules. It was found that Noc2-EYFP and Noc2(V58A)-EYFP colocalised strongly with ECFP-Rab27A, while Noc2(AAA)-EYFP adopted a more diffuse cytosolic localisation (Fig.4.4, A-I). However, as in previous experiments with the

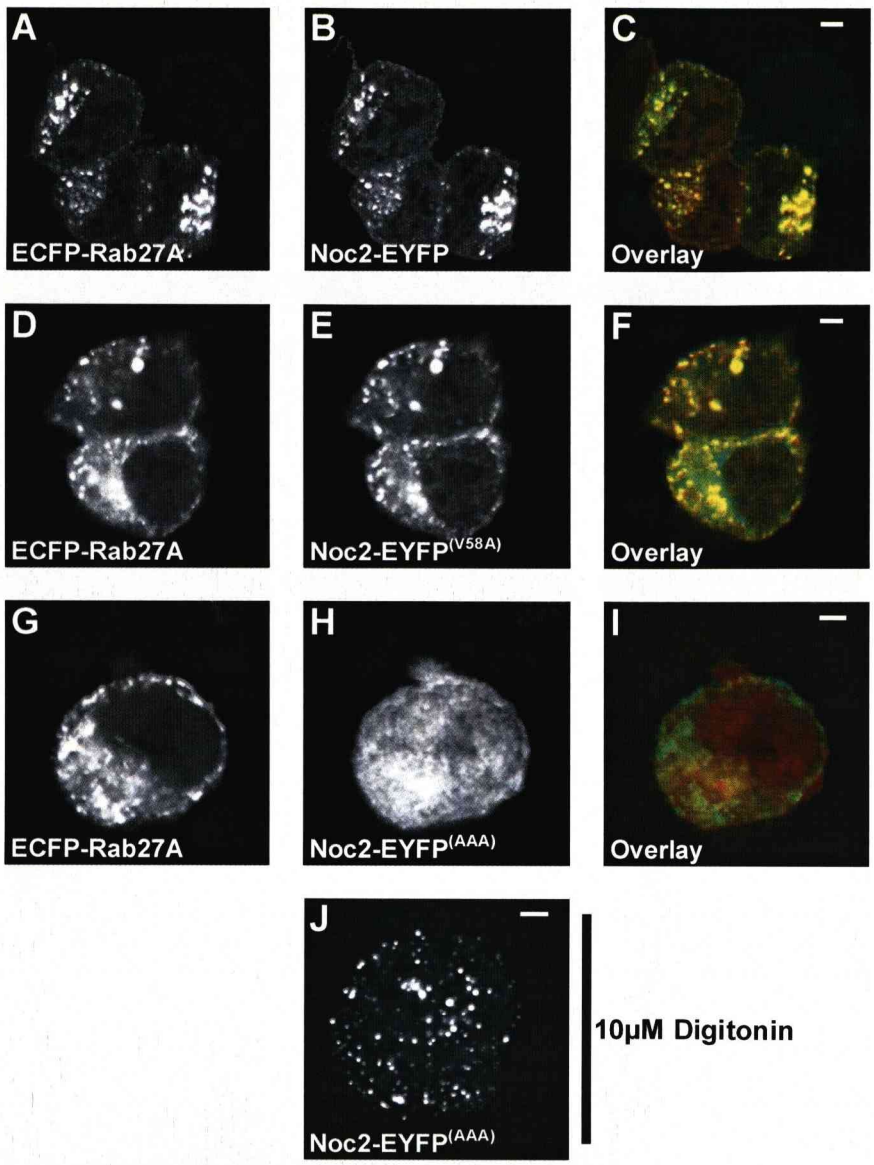
A
ECFP-Rab27A
B
Noc2-EYFP
C
Overlay
D
ECFP-Rab27A
E
Noc2-EYFP(V58A)
F
Overlay
G
ECFP-Rab27A
H
Noc2-EYFP(AAA)
I
Overlay
J
Noc2-EYFP(AAA)
10μM Digitonin
K
hGH release (% total)
30
25
20
15
10
5
0
ATP
- + - + - + - +
pEYFP-N1 (0.5μg)
Noc2-EYFP (0.5μg)
Noc2-EYFP V58A (0.5μg)
Noc2-EYFP AAA (0.5μg)
Total hGH
350
300
250
200
150
100
50
0
Noc2-EYFP (0.5μg)
Noc2-EYFP (0.5μg)
Noc2-V58A-EYFP (0.5μg)
Noc2-AAA-EYFP (0.5μg)

Figure 4.4. Effects of Noc2 V58A and SGAWFY→SGAAA mutation. (A-I) Cells were fixed 18 hours after cotransfection with plasmids encoding ECFP-Rab27, and Noc2-EYFP, Noc2(V58A)-EYFP or Noc2(AAA)-EYFP as indicated. Each overlay image on the right of the figure is a composite of green from the left image and red from the centre image. Areas of overlap appear in yellow. (J) Cells transfected with Noc2(AAA)-EYFP were exposed to a permeablisation buffer containing 10μM digitonin for 15minutes for the removal of free cytosolic protein prior to fixation. (K) Secretion of exogenous human growth hormone (hGH) from cells cotransfected with the above plasmids 48 hours before stimulation was assayed. Stimulated cells were exposed to 300 μM ATP, hGH release over 15 minutes was assayed and expressed as a percentage of total hGH (shown to the right of the figure). Data are expressed as mean ± s.e.m. (n=6). Bars, 4μm.

Rab27A mutants (see Fig.3.6, D-F), removal of cytosolic protein from cells through digitonin permeablisation prior to fixation, revealed a that small component of the Noc2(AAA)-EYFP was associated with punctae likely to be secretory granules (Fig.4.4, J). When the constructs were used in hGH cotransfection experiments, it was found that Noc2-EYFP inhibited hGH secretion in a manner comparable to wild type Noc2 (compare Fig.4.3, B and Fig.4.4, K), indicating that its fluorescent tag was unlikely to have affected its physiological activity. Cotransfection of Noc2(V58A)-EYFP produced a more moderate inhibitory effect, while Noc2(AAA)-EYFP, produced a smaller effect still. Together, these data are consistent with previous work suggesting that interaction with Rab proteins is critical for Noc2 function, and may support the suggestion that Noc2 is recruited to secretory vesicles via Rab27A rather than by Rab3A (Haynes *et al.*, 2001; Cheviet *et al.*, 2004a; Fukuda *et al.*, 2004).

4.2.3 Rab effector dynamics

As previously discussed, it is thought that many Rab effectors, including Rabphilin, Granuphilin and Noc2, are recruited to secretory granule membranes through their interaction with Rab proteins (Stahl *et al.*, 1996; Fukuda, 2003b; Fukuda *et al.*, 2004). In support of this suggestion, it is

found that mutations in these effectors that abolish their capacity to bind to Rab proteins, also markedly reduce that recruitment (Cheviet *et al.*, 2004a; Fukuda *et al.*, 2004). It was found here previously using FRAP experiments that the Rab proteins Rab3A and Rab27A show respectively dynamic and sustained interaction with secretory granules (Handley *et al.*, 2007). It was therefore decided to expand these experiments to include the Rab effectors, in order to identify any similar differences.

PC12 cells were transfected with vectors encoding the EGFP-tagged effectors. As before, 18 hours following transfection, cells expressing moderate levels of fluorescent protein were selected, regions of interest (ROIs) within these cells were bleached with high intensity laser, the recovery of fluorescence within these areas was recorded over time, and this data was then normalised with respect to total cell fluorescence to correct for general photobleaching. Example frames from these experiments are shown in Fig.4.5, A-L. Initial fluorescence in each experiment was normalised to 100 arbitrary fluorescence units in order that data from multiple bleaching experiments could be pooled. The pooled data for each construct is shown in Fig.4.5, M. In Fig.4.5, N, the same data has been normalised with respect to the first reading taken following bleaching. Fluorescence recovery due to diffusion of cytosolic fluorescent protein from unbleached regions of cells into the bleached region is likely to be largely complete at this point (~1.6 seconds post-bleach), and so the normalised data reflects recovery due to other sources. Recovery profiles of EGFP-Rab27A and EGFP-Rab3A are presented for comparison.

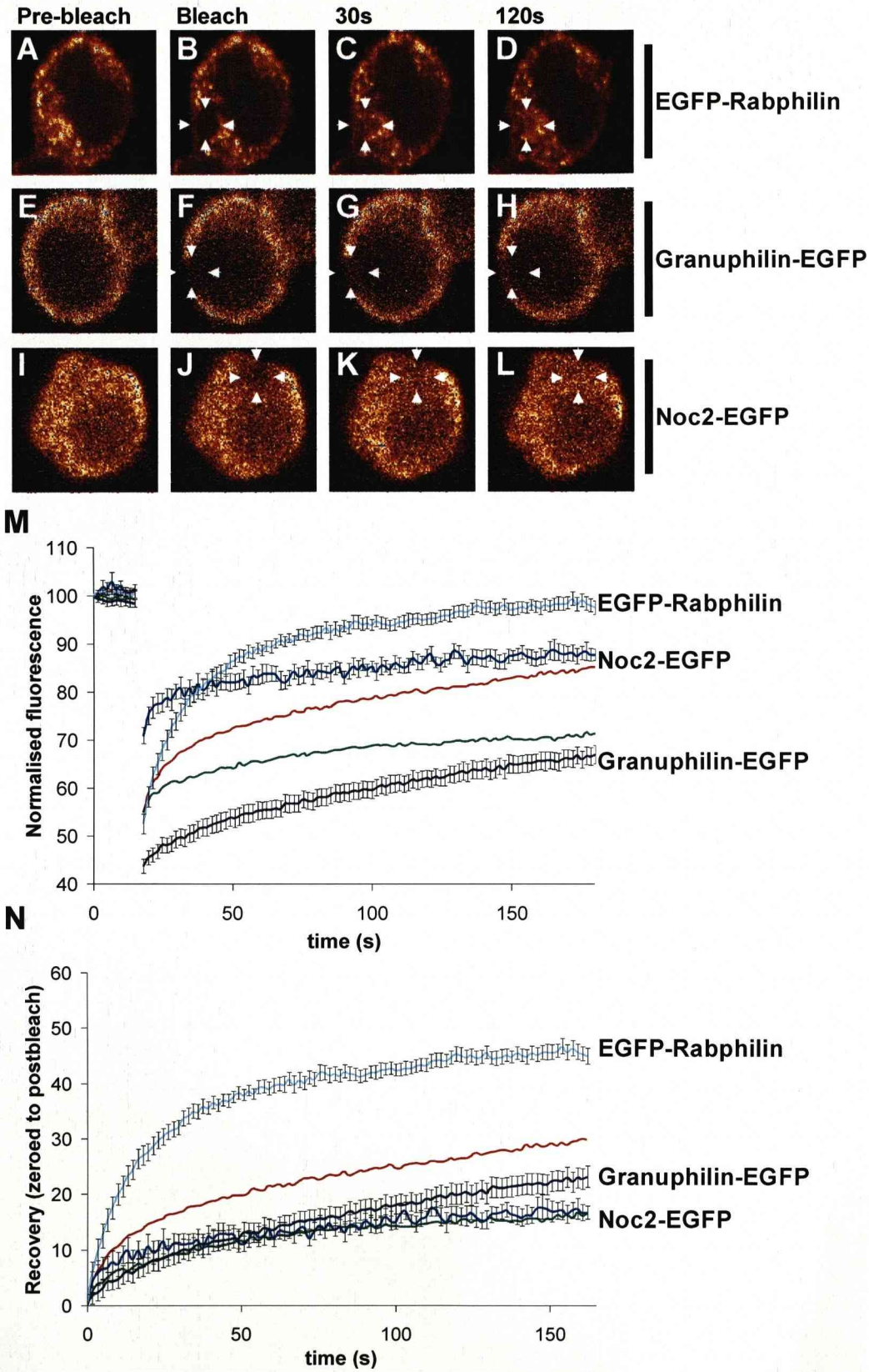

Figure 4.5. Dynamics EGFP-tagged Rab effectors on secretory granules in PC12 cells. (A-L) PC12 cells were transfected to express the indicated EGFP-tagged protein and imaged 18 hours following transfection. Regions of interest (ROIs) in each cell were bleached with high intensity laser (arrows) and the fluorescence recovery in these areas was recorded over time. [contd. on next page]

Figure 4.5 contd. (M) Plot of fluorescence recovery data corrected for general photobleaching and combined after initial fluorescence for each cell was normalised to 100. (N) Plot of fluorescence recovery as above but with first post-bleaching data point normalised to 0. Data shown are mean ±s.e.m. of 18 EGFP-Rabphilin, 21 Granuphilin-EGFP and 11 Noc2-EGFP expressing cells. Recovery profiles of EGFP-Rab3A (red) and EGFP-Rab27A (green) are provided for comparison.

When EGFP-Rabphilin-expressing cells were bleached, fluorescence levels recorded in bleached regions were found to be initially reduced to a similar extent to those recorded where EGFP-Rab3A and EGFP-Rab27A-expressing cells were bleached in previous experiments. This suggests that similar proportions of these exogenously expressed proteins are present in cytosolic and granule-associated fractions within cells (Fig.4.5, N-M). Fluorescence recovery of EGFP-Rabphilin following bleaching was rapid, and was almost complete within the timeframe of experiments (Fig.4.5, M). Since a large component of this fluorescence recovery is likely to be the result of exchange between bleached, granule-associated protein, and unbleached protein, this data indicates that like Rab3A, Rabphilin associates with granules in a dynamic manner. Indeed, in gross terms, it appears that EGFP-Rabphilin is exchanged more rapidly between the cytosol and granule membranes than EGFP-Rab3A. Further, the extent of its recovery indicates that most, if not all of the protein, is continually exchanged in this way.

The large reduction in Granuphilin-EGFP fluorescence that was recorded following bleaching suggests that a high proportion of the expressed protein is granule-associated, while the slow recovery of this fluorescence indicates that Granuphilin can associate with granules in a relatively sustained manner (Fig.4.5, M). The same bleaching protocol was applied

to Granuphilin-EGFP-transfected cells as was applied to cells transfected with other constructs. However, Granuphilin-EGFP bleaching data should not be directly compared to the other bleaching data presented here, because this construct labels a distinct population of peripheral secretory granules (Fig.4.2, D-F, J-L). Nevertheless, it may be possible to interpret the observed differences between the recovery profiles generated for Granuphilin-EGFP, and EGFP-Rab27A, which was previously suggested to show sustained interaction with granules (Handley *et al.*, 2007). Where the first few fluorescence readings following bleaching are compared, there is a comparatively fast, albeit very limited, recovery of EGFP-Rab27A fluorescence. This may be explained, if that recovery is related to the movement of unbleached granules at the border of bleached regions: since EGFP-Rab27A-labeled granules are distributed throughout the cytosol, more will usually border on these regions than would Granuphilin-EGFP-labeled granules, which are more spatially restricted. Where later fluorescence recovery is compared, recovery of Granuphilin-EGFP fluorescence is slightly faster than that of EGFP-Rab27A. This may indicate that some small level of exchange of bleached Granuphilin-EGFP, for unbleached protein, does occur.

Where Noc2-EGFP-transfected cells were imaged prior to bleaching, it was noted that the protein appeared more cytosolic than in previous experiments (compare Fig.4.5, I-L and Fig.4.2, G, P). This may reflect the use of fixed cells in those previous experiments, and it might also reflect the use of a wider confocal plane in the bleaching experiments. In any

case, the relatively small reduction in Noc2-EGFP fluorescence that was recorded following bleaching confirms the visual indication that a large proportion of the expressed protein is cytosolic in live cells (Fig.4.5, M). That fluorescence *is* reduced, almost certainly indicates that some of the protein associates with secretory granules. This protein is likely to do so in a sustained manner, since when fluorescence recovery not due to diffusion of cytosolic protein is compared, recovery profiles for Noc2-EGFP and EGFP-Rab27A are not significantly different (Fig.4.5, N).

Because the recovery profile of EGFP-Rabphilin indicated that the protein was subject to rapid exchange, it was decided to investigate the effects of a series of characterised Rabphilin mutations on this behavior. The generation of a series of mutants through site-directed mutagenesis of the existing EGFP-Rabphilin-encoding plasmid was attempted, and potential mutant plasmids were analysed by automated sequencing. A truncation mutant, Rabphilin(1-206) and a SGAWFF→SGAAA mutant, Rabphilin(AAA) were successfully generated (Fig. 2.6, B-C). However, the sequencing data showed that each mutant plasmid, and the original EGFP-Rabphilin plasmid, carried a deletion in the region encoding the N-terminus so that the 20th residue, an asparagine, became a lysine, and residues 21-24 became absent (N20K, Δ21-24). While this region is not suggested to contribute to the protein's interaction with Rab3A (Ostermeier and Brunger, 1999), future work may be required to determine whether the mutation affects Rabphilin dynamics. It should be noted that the localisation of the EGFP- Rabphilin(1-206) mutant in initial experiments

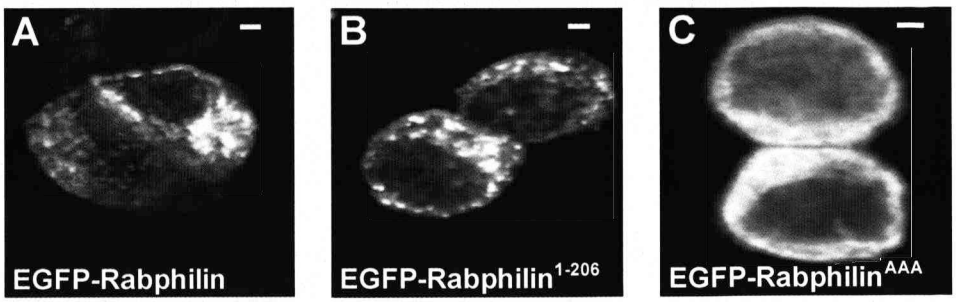

Figure 4.6. Effects of 1-206 truncation mutation and SGAWFF→SGAAAA Rabphilin mutation on EGFP-Rabphilin localisation in PC12 cells. (A-C) Cells were fixed 18 hours following transfection with a plasmid encoding the indicated fusion protein. Bars, 4μm.

was indistinguishable from that of the full-length protein, consistent with the protein's recruitment to secretory granules via the conserved N-terminal RBD (Fig.4.6, B)(Fukuda *et al.*, 2004). The EGFP-Rabphilin(AAA) mutant was largely cytosolic, consistent with the suggested requirement for the SGAWFF motif in interactions with Rab proteins (Fig.4.6, C) (Fukuda *et al.*, 2004).

4.2.4 Munc18-1 as a putative Rab3A effector

Effectors of Rab3A and Rab27A include members of the Synaptotagmin-like protein (Slp), and Synaptotagmin-like lacking C2 domains (Slac2), protein families (Fukuda, 2003a, 2005). These effectors possess homologous Rab-interacting domains (RBDs)(Fukuda, 2005). However, other effectors of these Rabs are identified that do not carry such domains. These include the calcium-binding protein Calmodulin, which could act as a Rab3A effector (Park *et al.*, 2002), and the 'priming factor' Munc13-4, which is suggested to act as a Rab27A effector (Shirakawa *et al.*, 2004; Neeft *et al.*, 2005). Recent work in this laboratory has suggested that the

Sec1/Munc18-like (SM) protein Munc18-1 might act as a Rab3A effector, and could therefore be included in this group.

It is thought that there is an essential requirement for an SM protein in every vesicular trafficking step in cells (Jahn, 2000; Burgoyne and Morgan, 2007). Further, it is thought that this requirement is related to the ability of these proteins to interact with the Syntaxin isoforms involved in each step (Burgoyne and Morgan, 2007). Munc18-1 is found to interact with Syntaxin1A at the plasma membrane (Rickman *et al.*, 2007). It is found to be essential for neurotransmission (Verhage *et al.*, 2000), and is suggested to be involved in the tethering/docking of synaptic vesicles in neurones (Weimer *et al.*, 2003), and that of secretory granules in neuroendocrine cells (Voets *et al.*, 2001b). It is also suggested to be involved in the later stages of exocytosis (Fisher *et al.*, 2001; Ciufo *et al.*, 2005).

A link between the function of Rab proteins and that of SM proteins was suggested by studies conducted in yeast, in which a point mutation in the SM protein Sly1p was found to suppress the effects of deletion of Ypt1, a Rab protein involved in ER to Golgi membrane trafficking (Dascher *et al.*, 1991; Ossig *et al.*, 1991). In later work in this laboratory, overexpression of a corresponding Munc18-1 mutant, E466K, in adrenal chromaffin cells, was found to increase the frequency of exocytotic events recorded following stimulation (Ciufo *et al.*, 2005).

More recent work in this laboratory, conducted by Graham *et al.*, 2008, has explored the basis for the above finding. Another mutation was introduced into the Munc18-1(E466K)-encoding sequence to generate a sequence encoding Munc18-1(E466K/R39C). In difference to the single mutant, it was found that overexpression of the double mutant did not enhance exocytotic responses in adrenal chromaffin or PC12 cells. Since the R39C mutation had been found to cause reduced interaction between Munc18-1 and the 'closed' conformation of Syntaxin (Fisher *et al.*, 2001; Ciufo *et al.*, 2005), that data suggested that this interaction was required for the enhancement produced by the single mutant. Pull-down experiments were conducted, but it was found that the binding of Syntaxin from bovine brain to GST-Munc18-1(E466K), and to GST-Munc18-1(E466K, R39C), was similar to that of unmutated GST-Munc18-1. On further analysis however, these experiments also showed that binding of Rab3A to these mutant proteins was increased. When direct binding assays were carried out, it was found that recombinant Rab3A interacted with both GST-Munc18-1, and GST-Munc18-1(E466K, R39C), either in the presence of GTPγS, or in the presence of GDPβS (Graham *et al.*, 2008).

Because Munc18-1(E466K) was found to interact with Rab3A from bovine brain lysate, it might also do so in PC12 cells. In order to examine whether such an interaction could affect the previously characterised dynamic association between Rab3A and secretory granules (Handley *et al.*, 2007), a series of FRAP experiments were carried out. Cells were transfected to express EGFP-Rab3A alone, or to coexpress EFGP-Rab3A and Munc18-

1(E466K). As previously, recovery of EGFP-Rab3A fluorescence in bleached regions was recorded over time. It was found that the recovery profile of EGFP-Rab3A was not significantly changed with coexpression of Munc18-1(E466K) (Fig.4.7, A). However, it was noted that the distribution of EGFP-Rab3A became more peripheral in a proportion of cells expressing this protein.

Previous work has implicated both Rab3A and Munc18-1 in tethering/docking of secretory granules to the plasma membrane (Voets *et al.*, 2001b; Tsuboi and Fukuda, 2006a). The newly identified direct interaction between these proteins, and the genetic interaction between homologous proteins (Dascher *et al.*, 1991; Ossig *et al.*, 1991), might suggest that they are components of the same tethering/docking machinery. Indeed, it has very recently been found that enhanced tethering/docking seen where Rab3A is overexpressed is dependent on expression of Munc18-1 (van Weering *et al.*, 2007). Given this data, the observations made during the imaging experiments might suggest that the enhancement in exocytotic responses, seen where Munc18-1(E466K) is overexpressed, is the result of some concomitant enhancement in the tethering/docking process.

In order to test the above suggestion, it was decided to assess the effects of overexpression of wild type and mutant Munc18s on the localisation of EGFP-Rab3A labeled granules in PC12 cells more quantitatively. Cells were transfected to coexpress a Munc18-1 protein, and EGFP-Rab3A,

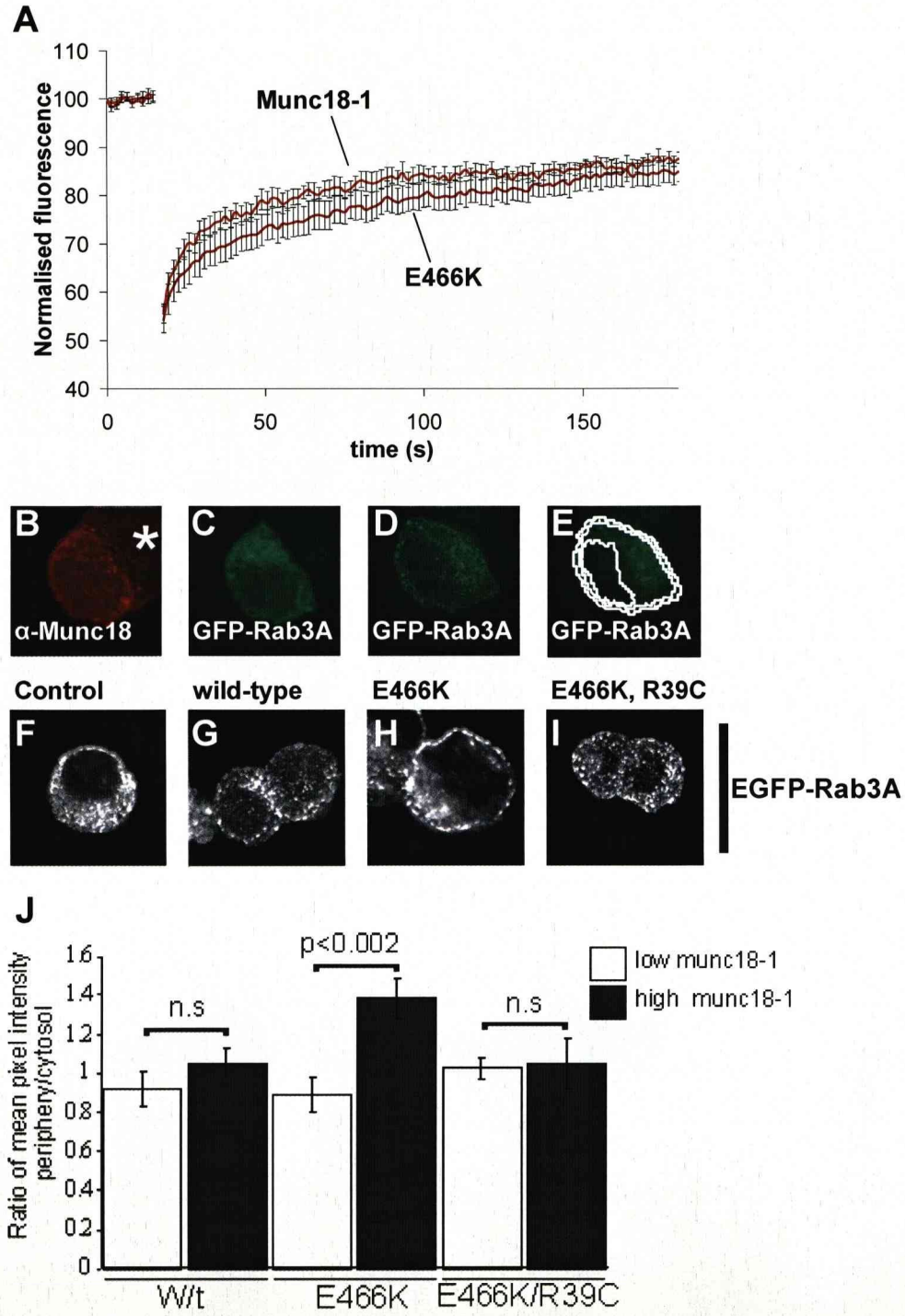

Figure 4.7. Effects of Munc18-1(E466K) coexpression on EGFP-Rab3A in PC12 cells. (A) PC12 cells were transfected to express EGFP-Rab3A alone, or EGFP-Rab3A and Munc18-1(E466K), and imaged 18 hours following transfection. Regions of interest (ROIs) in each cell were bleached with high intensity laser and the fluorescence recovery in these areas was recorded over time. Plot shows fluorescence recovery data, corrected for general photobleaching, and combined after initial fluorescence for each cell was normalised to 100. Data shown are mean ±s.e.m. of 10 control cells and 7 Munc18-1(E466K)-expressing cells. (B-C) Cells were transfected to coexpress EGFP-Rab3A and Munc18-1 and immunostained with anti-Munc18. Cells expressing exogenous Munc18-1 are identifiable because of greater staining "*" marks an untransfected cell. (D-E) For quantification of EGFP-Rab3A localisation, regions of interest were drawn around cell periphery and nucleus. [contd. on next page]

Figure 4.7 contd. (F-I) Localisation of EGFP-Rab3A in control cells, and cells expressing high levels of the indicated Munc18-1 protein. (J) Quantification of the ratio of mean pixel intensity at the cell periphery compared to cytosol. Cells on each coversilp were selected for high or background levels of Munc18-1 expression. The data are mean ±s.e.m, derived from 10 cells for each condition.

fixed 18 hours following transfection, and immunostained with an antibody against Munc18. When these cells were visualised, immunostained cells overexpressing a Munc18-1 protein were clearly distinguishable from those expressing only endogenous Munc18, as a result of their higher fluorescence intensity (compare adjacent cells in Fig.4.7, B-C). Cells expressing low levels of EGFP-Rab3A (see Fig. 2.7, D), and either low or high levels of each Munc18-1, were imaged. In order to generate a numerical measure for the extent of peripheral localisation of EGFP-Rab3A in each cell, regions of interest were drawn defining peripheral, cytosolic and nuclear areas (see Fig.4.7, E). For each image, mean pixel intensities in the defined peripheral and cytosolic areas were determined, and these values were then expressed as a ratio. Fig.4.7, F-I, shows representative images of EGFP-Rab3A localisation in cells expressing high levels of the Munc18-1 protein indicated. Fig.4.7, J, shows the pooled data from these experiments. As shown, it was found that overexpression of Munc18-1(E466K) caused significant redistribution of EGFP-Rab3A-labeled granules to the periphery of cells, while the overexpression of wild type Munc18-1, or Munc18-1(E466K, R39C), had no effect.

In the experiments described above, it was possible that the apparent increase in peripheral granule numbers caused by Munc18-1(E466K) overexpression was dependent on the simultaneous overexpression of

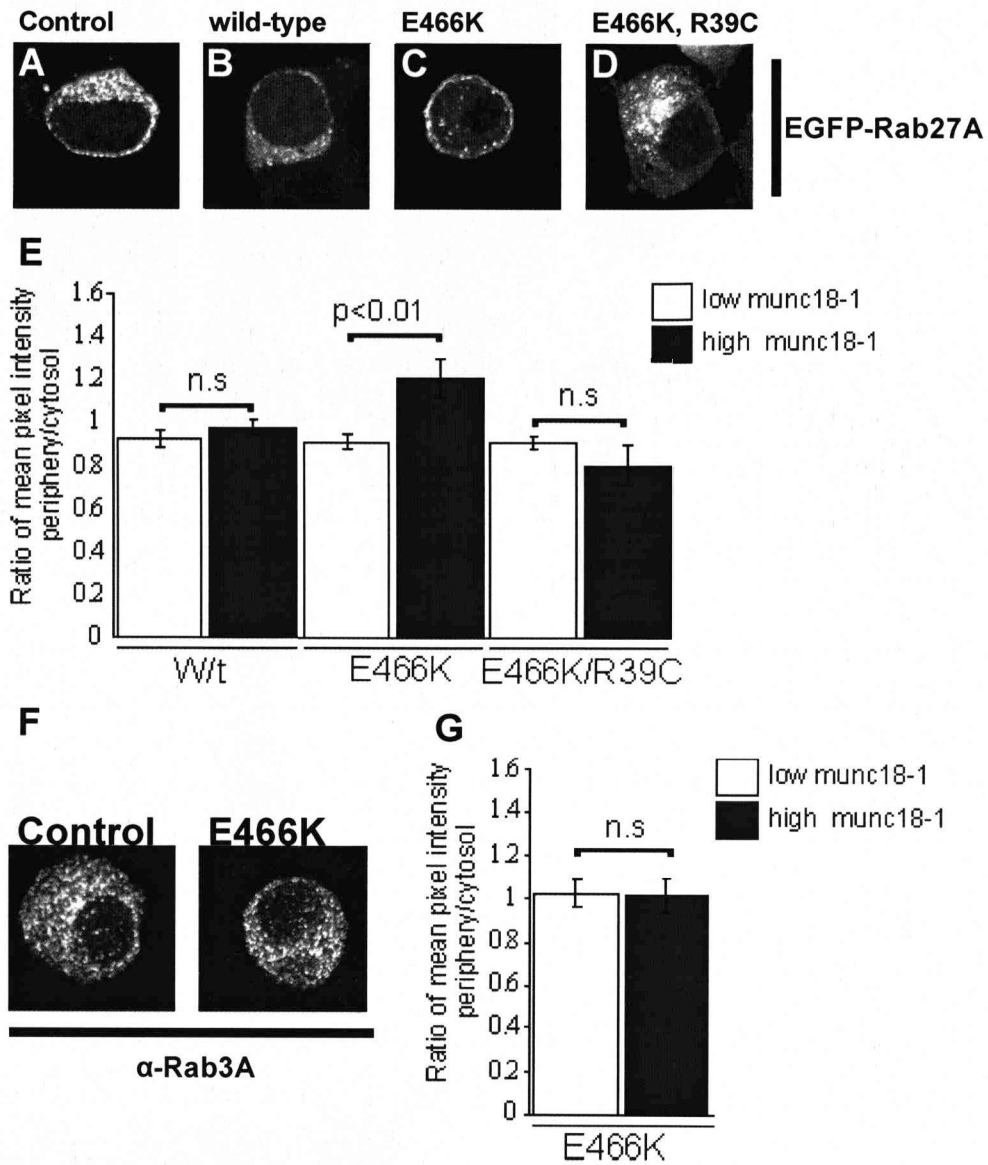

Figure 4.8. Effects of Munc18-1(E466K) expression on EGFP-Rab27A, and endogenous Rab3A, in PC12 cells. (A-D) Localisation of EGFP-Rab27A in control cells, and cells expressing high levels of the indicated Munc18-1 protein. (E) Quantification of the ratio of mean EGFP-Rab27A pixel intensity at the cell periphery compared to cytosol, in control cells and cells expressing the indicated Munc18-1 protein (F) Localisation of endogenous Rab3A in control cells, and cells expressing high levels of Munc18-1(E466K). (G) Quantification of the ratio of mean α-Rab3A pixel intensity at the cell periphery compared to cytosol, in control cells and cells expressing the indicated Munc18-1 protein. Cells on each coversilp were selected for high or background levels of Munc18-1 expression. The data are mean ±s.e.m, derived from 10 cells for each condition.

EGFP-Rab3A. In order to address this possibility, additional experiments were carried out in which cells were transfected to coexpress each munc18-1 protein, and EGFP-Rab27A (Fig.4.8, A-E). It was found that

overexpression of Munc18-1(E466K) caused a redistribution of EGFP-Rab27A-labeled granules similar to that of EGFP-Rab3A-labeled granules. As before, overexpression of the other Munc18-1s had no effect.

Since both EGFP-Rab3A, and EGFP-Rab27A, predominantly label relatively newly synthesised secretory granules (Handley *et al.*, 2007), it was possible that the effects of Munc18-1(E466K) overexpression were selectively exerted on this granule population. This possibility was addressed by transfecting cells with Munc18-1(E466K), and comparing the distribution of endogenous Rab3A in transfected, and untransfected cells (Fig.4.8, F-G). No differences in granule distribution were identified in these experiments.

The experiments described above, in which overexpression of Munc18-1(E466K) was shown to redistribute EGFP-Rab3A and EGFP-Rab27A-labeled secretory granules to the periphery of cells, indicate that this protein potentiates the tethering/docking of these granules at the plasma membrane. The fact that overexpression of Munc18-1(E466K, R39C) did not produce such redistribution, is a strong indication that direct interaction between Munc18-1(E466K), and the 'closed' conformation of Syntaxin, is required for this potentiation. However, elsewhere, Semliki forest virus (SFV)-mediated Munc18-1 overexpression has been suggested to increase numbers of tethered granules indirectly, through disruption of cortical actin (Toonen *et al.*, 2006). The effects of Munc18-1(E466K) overexpression on cortical actin were therefore investigated. As previously,

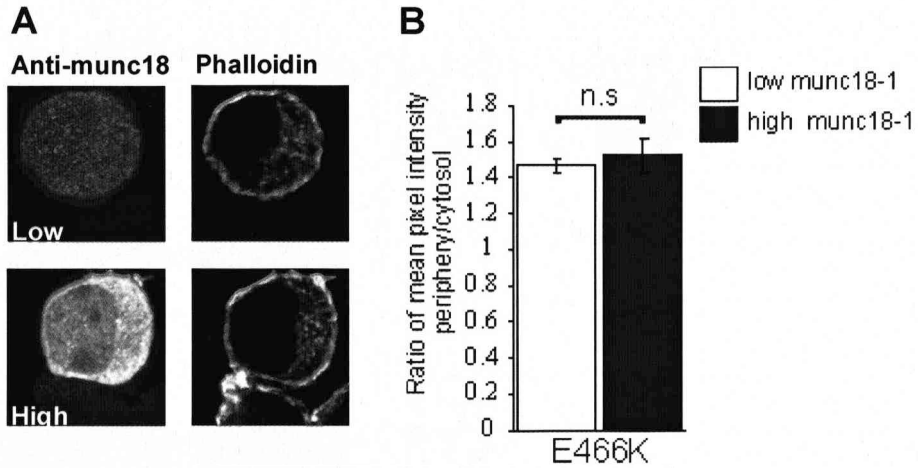

Figure 4.9. Effects of Munc18-1(E466K) expression on cortical actin in PC12 cells. (A) Phalloidin staining in control cells, and cells expressing high levels of Munc18-1(E466K). (B) Quantification of the ratio of mean phalloidin pixel intensity at the cell periphery compared to cytosol, in control cells and cells expressing Munc18-1(E466K). Cells on each coversilp were selected for high or background levels of Munc18-1 expression. The data are mean ±s.e.m, derived from 10 cells for each condition.

Munc18-1(E466K)-transfected cells were fixed and immunostained. Cortical actin was labeled with a phalloidin conjugate. No difference was observed in the distribution of cortical actin in transfected as compared to untransfected cells (Fig.4.9). This data further supports the suggestion that Munc18-1(E466K) participates in tethering/docking directly.

4.3 Discussion

This chapter has described a series of experiments designed to further characterise the known exocytotic Rab effectors Rabphilin, Granuphilin, Noc2 and a novel interactor Munc18-1. In the course of these experiments, each effector has shown distinct properties, and so this discussion will focus on the possible structural basis for, and functional implications of, each of these differences with respect to each effector in turn.

4.3.1 Rabphilin

The findings showing that exogenous EGFP-Rabphilin localises to secretory granules in PC12 cells (Fig.4.2, A-C, J-L) are consistent with previous reports suggesting that the effector is recruited by Rab proteins associated with these structures (Fukuda *et al.*, 2004). Since the Rabphilin(AAA)-EGFP mutant, which is unable to interact with Rab proteins, was found to be largely cytosolic (Fig.4.6, C), it is unlikely that other interactions, such as that with the phospholipid phosphatidylserine (PS)(Yamaguchi *et al.*, 1993; Montaville *et al.*, 2007), are sufficient to mediate such recruitment. It has been suggested that Rabphilin interacts physiologically with both Rab3 and Rab27 isoforms (Fukuda *et al.*, 2004; Deak *et al.*, 2006). Because a 5-10x stronger interaction is seen with Rab27A, than with Rab3A in vitro, it is thought that Rabphilin preferentially interacts with Rab27A in PC12 cells, which express both Rabs (Fukuda *et al.*, 2004). It is therefore surprising that like EGFP-Rab3A, but in contrast to EGFP-Rab27A (Fig.3.7), EGFP-Rabphilin was shown by FRAP

experiments to associate with secretory granules in a dynamic manner (Fig.4.5, M). In fact, the recovery profile of EGFP-Rabphilin indicates that the rate of its exchange between cytosolic and granular localisations was even faster than that of EGFP-Rab3A. Further, recovery was virtually complete, indicating that most, if not all, of the cellular compliment of the protein was subject to this exchange.

One possible explanation for a dynamic interaction between EGFP-Rabphilin and granular Rab proteins might be that the effector has a relatively low binding affinity for these Rabs, and so is susceptible to continual displacement by other effectors. Indeed, a previous study has suggested that the differing affinities of the Rab27A effectors Slp2a and Melanophilin for this Rab are important for their sequential binding, and their sequential function in melanosome transport (Fukuda, 2006). However, in this case, this explanation is unlikely, since Rabphilin has been shown to bind to Rab27A with very high affinity, and with higher affinity than either Granuphilin or Noc2, which were shown here to exhibit a sustained interaction with granules (Fukuda, 2006)(Fig.4.5, M-N). The FRAP data presented here might support the suggestion that the interaction between Rab27A and Rabphilin is stronger than that between Rab27A and Noc2 in vivo, since EGFP-Rabphilin fluorescence immediately following bleaching is much lower than Noc2-EGFP fluorescence indicating that a larger proportion of expressed EGFP-Rabphilin is granule-associated (Fig.4.5, M-N). While it also appears from the FRAP data that an even larger proportion of expressed Granuphilin-

EGFP is granule-associated, it is possible that this finding is not solely related to the affinity of Granuphilin for Rab27A (see 4.3.2 'Granuphilin' below).

Since Rabphilin is known to bind only GTP-bound Rab protein (Stahl *et al.*, 1996; Fukuda *et al.*, 2004), it is possible that its disassociation from Rab proteins is coupled to their hydrolysis of bound GTP. In support of this hypothesis, antibodies against Rabphilin can coimmunoprecipitate Rab3A and Rab27A from PC12 cell extracts in the presence of GTPγS, indicating that interaction between Rabphilin and the 'active' forms of these proteins must be relatively stable (Fukuda *et al.*, 2004). Further, while the effects of Rabphilin on the intrinsic GTPase activity of Rab27A are unknown, it is notable that the protein has been reported to show a weak GAP activity towards Rab3A in vitro (Kishida *et al.*, 1993). Thus, binding of Rabphilin to a GTP-bound Rab protein might potentiate GTP hydrolysis and the effector's subsequent dissociation from the resulting GDP-Rab; Rabphilin might mediate its own rapid association and dissociation from granules. While the RBD1 domain of Noc2 or Granuphilin, which do not show such rapid association and dissociation, is found to be sufficient for interaction with GTP-Rab27A, Rabphilin requires its RBD1 and RBD2 domains as well as an intervening cysteine-rich region for this interaction (Fukuda, 2003b; Fukuda *et al.*, 2004). Speculatively, this requirement might be linked to the structural basis for a GAP activity not identified in the other effectors.

Whatever the reason for the transience of the Rab-Rabphilin interaction, its possible functional importance should be considered. Rabphilin has been variously suggested to function through binding to factors including β-adducin, GTP cyclohydrolase I, Rabaptin5 and AnnexinA4 (Imazumi *et al.*, 1994; Miyazaki *et al.*, 1994; Ohya *et al.*, 1998; Willshaw *et al.*, 2004). However, most interest has surrounded its identified interactions with the SNARE protein SNAP-25, and the actin-bundling protein α-actinin (Kato *et al.*, 1996; Baldini *et al.*, 2005; Tsuboi and Fukuda, 2005; Deak *et al.*, 2006; Tsuboi *et al.*, 2007).

Binding of Rabphilin to SNAP-25 has been suggested to enhance tethering/docking and increase fusion events in response to stimulation (Tsuboi and Fukuda, 2005; Tsuboi *et al.*, 2007). TIRF microscopy showed that where wild type Rabphilin was overexpressed, there was an increased number of NPY-Venus labeled granules in the evanescent field very close to the plasma membrane of cells, and an increase in NPY-Venus-associated release events on exposure of cells to high K^+ (Tsuboi and Fukuda, 2005). In contrast, overexpression of a Rabphilin mutant unable to interact with SNAP-25 led to a reduction in granules at the plasma membrane and reduced release events (Tsuboi *et al.*, 2007). The authors of these studies have speculated that Rabphilin binding to SNAP-25 promotes initial docking/tethering, while an additional activity of the protein might promote fusion. However, the data they present might also suggest a role for Rabphilin earlier in the exocytotic pathway. They show that overexpressed Rabphilin increased the number of docked/tethered

granules prior to stimulation, and increased release events expressed as a proportion of this number, but also show that the numbers of docked/tethered granules following stimulation are similar to those prior to stimulation (Tsuboi and Fukuda, 2005). These data indicate that Rabphilin must enhance mobilisation of granules towards the plasma membrane, either to replenish the pool of docked/tethered granules, or to contribute to the enhanced exocytotic response. Since the proportion of the granules that were docked/tethered prior to stimulation that actually underwent fusion was not reported, the extent of the contribution to the exocytotic response from granules entering the evanescent field during stimulation in these experiments was not clear. However, it should be noted that the probability of release from initially mobile undocked/cytoplasmic granules is reported to be substantially higher than that from initially static docked/tethered granules in PC12 cells (Han, 1999).

It is reported that the association between secretory granules and cross-linked F-actin is enhanced in the presence of Rabphilin (Baldini *et al.*, 2005). It is also reported that binding of Rabphilin to α-actinin enhances the cross-linking activity of α-actinin towards such actin filaments (Kato *et al.*, 1996). These data have been interpreted as suggesting that Rabphilin facilitates actin remodelling, and the localisation of granules to F-actin-rich areas, such as the cortical regions of PC12 cells (Kato *et al.*, 1996; Baldini *et al.*, 2005). However, the findings presented here, indicating that Rabphilin interacts transiently with Rab proteins, and those discussed above, indicating that the effector enhances mobilisation of granules, might

add a new dimension to such interpretation. The transient interaction between Rabphilin and Rab proteins might be seen as implying that the protein functions, through α-actinin, in the local remodelling of actin filaments surrounding cortical granules, rather than in their attachment to this actin. The enhanced mobilisation of granules where Rabphilin is overexpressed could be the result of such remodelling.

4.3.2 Granuphilin

Overexpressed Granuphilin-EGFP was found to predominantly label granules at the periphery of PC12 cells (Fig.4.1, C). Unlike EGFP-Rabphilin or Noc2-EGFP, it did not appear to colocalise completely with mRFP-tagged Rab3A or Rab27A, but instead labeled an overlapping pool of granules (Fig.4.2, D-F, M-O). A possible basis for this difference might be the characterised interactions of the protein with Munc18 and/or Syntaxin isoforms, and/or Phosphatidylinositol (4,5)-bisphosphate (PIP_2)(Coppola *et al.*, 2002; Torii *et al.*, 2002; Torii *et al.*, 2004; Fukuda *et al.*, 2005; Gomi *et al.*, 2005; Tsuboi and Fukuda, 2006b; Osborne *et al.*, 2007). Granuphilin can bind to Munc18 in a manner independent of any interaction with Rab proteins (Fukuda *et al.*, 2005), while Munc18 can bind Syntaxin at the plasma membrane (Rickman *et al.*, 2007). Some reports have suggested that Granuphilin can bind Syntaxin directly (Torii *et al.*, 2002; Torii *et al.*, 2004). The binding of Granuphilin to PIP_2 is less well defined, but like Syntaxin, this lipid is localised to the plasma membrane (Osborne *et al.*, 2007). These interactions might suggest that Granuphilin is targeted to peripheral granules because it is here that the protein can

stably bind to both Rab proteins present on secretory granules, and to its other binding partners present on the plasma membrane. Indeed, such a targeting mechanism has been described previously for other Rab effectors (Pfeffer, 2003). For example, the Rab9 effector TIP47, which also binds to mannose-6-phosphate receptor (M6PR), is preferentially targeted to late endosomes, where both of these proteins are present (Carroll *et al.*, 2001). In this case Rab9 and M6PR are suggested to constitute a 'two-component address' (Pfeffer, 2003).

The FRAP data presented here might provide some support for the above hypothesis. The lower recorded fluorescence for Granuphilin-EGFP than for Noc2-EGFP following bleaching and recovery of cytosolic fluorescence (compare initial post-bleaching datapoints in (Fig.4.5, M) shows that a greater proportion of expressed Granuphilin-EGFP is granule-associated, even though Granuphilin and Noc2 are found to bind to Rab27A with similar affinity (Fukuda, 2006). While there are other explanations for this finding, one explanation is that as suggested, Granuphilin-EGFP localisation is stabilised through interaction with both Rab27A on granules, and another binding partner at the plasma membrane. It should be noted here that the FRAP recovery profiles of Granuphilin-EGFP and Noc2-EGFP, like that of EGFP-Rab27A, indicate that where these proteins do interact with granules, they do so in a relatively sustained manner (Fig.4.5, N). Since the localisation of EGFP-Granuphilin is different from that of the EGFP-tagged Rab proteins and other effectors, its recovery profile may not be directly comparable to theirs. However, it does appear that Granuphilin-

EGFP fluorescence recovery occurs at a slightly faster rate than that of either Noc2-EGFP or EGFP-Rab27A. An explanation of this finding might be that while stable interactions between granule membrane, Granuphilin-EGFP, and plasma membrane can exist, granular and plasma membranes may occasionally be drawn apart, releasing bleached Granuphilin-EGFP, and granular and plasma membranes may be occasionally drawn together, creating optimal binding sites for unbleached protein.

As may be inferred from the above discussion, Granuphilin is widely suggested to be involved in the tethering/docking of secretory granules to the plasma membrane (Torii *et al.*, 2002; Torii *et al.*, 2004; Tsuboi and Fukuda, 2006b). Its overexpression is found to increase numbers of peripheral granules in PC12 cells and Min6 cells (this study Fig.4.2, D-F, M-O)(Torii *et al.*, 2004; Tsuboi and Fukuda, 2006b), while the numbers of such granules are reduced in its absence (Gomi *et al.*, 2005). It is likely that multiple Granuphilin molecules are required to mediate granule tethering, because of the relatively large size of these organelles, and the likely kinetic forces to which they are subject. Therefore, tethering may be potentiated if the protein's binding partners are arranged in clusters. Indeed, this may well be the case. Rab proteins have already been shown to form clustered 'domains' on other membrane compartments (Zerial and McBride, 2001), and Rab27A may do so on secretory granules (see Discussion in Chapter 3). At the same time, clusters of syntaxin molecules are identified at the plasma membranes of PC12 and Min6 cells (Lang *et al.*, 2001; Ohara-Imaizumi *et al.*, 2004).

If closely apposed Rab and Munc18/Syntaxin/PIP_2 clusters can create a microenvironment with high affinity for Granuphilin, and thereby facilitate tight association between secretory granules and the plasma membrane, what is the physiological purpose of this process? It has been suggested that Granuphilin acts to constrain stimulation-evoked fusion, because while its overexpression increases numbers of docked/tethered granules at the plasma membrane, it also reduces the size of exocytotic responses (Torii *et al.*, 2002; Tsuboi and Fukuda, 2006b). This reduction may be due to the formation of complexes that reduce the probability of SNARE engagement, docking and fusion. It was found in one study that Granuphilin could bind to Syntaxin2/3 only in a Munc18-2-dependent manner, and that the interaction was reduced when the L165A/E166A 'open' mutant of Syntaxin was expressed (Fukuda *et al.*, 2005). In another study, Granuphilin could not bind to 'open' Syntaxin1a (Torii *et al.*, 2002). This data suggests that Granuphilin might be involved in sequestering 'closed' Syntaxin, and preventing its incorporation into functional SNARE complexes. Such sequestration might limit the fusion-competence of Granuphilin-tethered Granules, and may also limit the availability of 'free' Syntaxin at the plasma membrane, thereby reducing the likelihood of *any* granule undergoing fusion.

Where Granuphilin was moderately overexpressed in hGH cotransfection experiments carried out here, no effect on ATP-stimulated hGH secretion was seen (Fig.4.3, A). It may be that an increase in fusion-incompetent

granules at the plasma membrane has a limited effect on exocytotic responses, while these responses are reduced only where enough Granuphilin is present to severely limit 'free' Syntaxin. This might be considered likely, since as previously noted, initially undocked/cytoplasmic granules in PC12 cells make a very substantial proportionate contribution to exocytotic responses in PC12 cells (Han, 1999). It has been found that in the absence of Granuphilin, cellular levels of Syntaxin and Munc18 are reduced, while exocytotic responses are enhanced (Gomi *et al.*, 2005). This might be seen as suggesting that an important physiological function of Granuphilin is in the stabilisation of these proteins, as well as in their sequestration.

In adrenal chromaffin cells, it has been found that while granules can remain tethered at the plasma membrane for extended periods of time, older granules are usually found in the cell interior, away from the membrane (Duncan *et al.*, 2003). This could suggest that physiological Granuphilin-mediated tethering is reversible, a suggestion that might also be supported by the recovery profile of Granuphilin-EGFP described here (see above). Speculatively, a mechanism for Granuphilin complex disassembly might involve Rab27A GTP-hydrolysis or might involve its binding to membrane lipids. While Granuphilin interacts with both GTP and GDP bound Rab27A, different regions are involved in this binding. RBD1 is necessary and sufficient to bind to GTP-Rab27A, while RBD2 is required for the interaction with GDP-Rab27A, since mutations of residues in the SGAWFY motif can selectively abolish this interaction (Fukuda, 2003b).

Therefore, the conformation of the Granuphilin may differ according to the nucleotide-bound state of associated Rab27A, and this may regulate its binding to other factors. While Granuphilin interacts with Munc18 isoforms via a central 'linker' domain between its N-terminal Rab-interacting domain and C-terminal C2 domains (Tsuboi and Fukuda, 2006b), it is likely that one or both of its C2 domains are required for its possible interaction with PIP_2. It has been found elsewhere that stimulated exocytosis is associated with the local phospholipase C (PLC)-catalysed breakdown of PIP_2 at exocytotic sites (Robert Woolley, Personal Communication). Loss of any PIP_2 interaction might also lead to conformational change and altered binding to other factors.

4.3.3 Noc2

The findings presented here showing that fluorescently labeled Noc2 colocalises with fluorescently labeled Rab27A (Fig.4.2, G-I, Fig.4.4, A-C), and that this colocalisation is largely abolished in the Rab-binding deficient 'AAA' mutant but unaffected by the Rab3A-binding deficient V58A mutant (Fig.4.4, D-J) are consistent with reports that the protein is recruited to granules through interaction with Rab27A (Fukuda *et al.*, 2004). Another finding that might support this suggestion is that the FRAP recovery profile of Noc2-EGFP is similar to that of EGFP-Rab27A, while EGFP-Rab3A recovery is faster than that of these proteins (Fig.4.5, N). This finding, indicating that Noc2 interacts with granular Rab27A in a sustained manner, might be explained in several ways. Apart from Granuphilin (Fukuda, 2003b), most Rab effectors are thought to interact with GTP-bound Rab

proteins preferentially, and the interaction between Noc2 and GTP-Rab27A at the granule membrane may be stable. However, in one study, Noc2 appears to interact with GDP-Rab27A more strongly than with GTP-Rab27A (Cheviet *et al.*, 2004a), and this interaction might also be stable. This suggestion would be controversial, since Rab27A(T23N), a mutant that is largely GDP-bound, shows reduced binding to Noc2 (Fukuda *et al.*, 2004). However, it is possible that this is a direct result of the mutation, rather than of the mutant's nucleotide binding.

Whatever the specifics of Noc2 recruitment to granules, a major finding reported here and elsewhere is that moderate Noc2 overexpression can inhibit regulated secretion (Fig.4.3, B, Fig.4.4, K)(Haynes *et al.*, 2001; Cheviet *et al.*, 2004a). Since the interaction between Noc2 and Rab27A appears to be sustained, overexpression might produce such inhibition by saturating available Rab27A binding sites, and thereby reducing their availability to other effectors. However, it was found here that the Noc2(V58A) mutant produced less inhibition than the unmutated protein (Fig.4.4, K)(Haynes *et al.*, 2001), despite its reportedly equivalent binding to Rab27A (Cheviet *et al.*, 2004a). That might reflect a functional deficit in the mutant protein since the V58 residue of Noc2 is thought to be involved in interactions with the 'switch' regions of Rab proteins and may therefore be involved in functional transduction of nucleotide binding information (Haynes *et al.*, 2001). It is also relevant that the 'AAA' Noc2 mutant produced a small but significant inhibition of secretion, even though it barely bound to granules (Fig.4.4, H, J, K).

The function of Noc2 is relatively poorly characterised, and so it is difficult to say why its activity should reduce secretion. However, the existing literature may provide some clues. Experiments on a Noc2 knockout mouse have suggested that the protein may be involved in the suppression of signalling downstream of G_i in pancreatic β-cells, in a manner dependent on its interaction with Rab proteins (Cheviet *et al.*, 2004b; Matsumoto *et al.*, 2004). A mechanism for this activity has not been uncovered. However, it has been found in another study that increased protein kinase A (PKA) activity in a β-cell line can lead to the transcriptional repression of Rab3A, Rab27A, Granuphilin and Noc2 (Abderrahmani *et al.*, 2006). Since Noc2-mediated suppression of G_i might potentiate PKA activity, the endogenously expressed protein may exquisitely regulate the level of its own expression, and that of the other proteins mentioned, through negative feedback. Where Noc2 is overexpressed on a non-native promoter, however, the expression of these other proteins might be reduced, as the feedback system is dysregulated.

The timescale on which PKA-mediated reduction in Rab and effector protein levels in β-cells operates, might be consistent with the suggestion that overexpressed Noc2 might have reduced levels of these proteins in PC12 cells in the hGH cotransfection experiments carried out in this study (Abderrahmani *et al.*, 2006). However, it has been found elsewhere that acute treatment of permeablised parotid acinar cells, with an antibody that

disrupts the interaction between Noc2 and Rab proteins, reduces the isoproterenol-stimulated secretory responses of these cells (Imai *et al.*, 2006). This indicates that Noc2 is likely to participate directly in the secretory process, as the effects of the antibody cannot be due to any effect Noc2 has at the transcriptional level. It is possible that this participation might involve G_i-suppression, however, since isoproterenol is a β-adrenoceptor agonist, and β-adrenoceptor activation is linked to G_i and G_s signalling in parotid cells (Watson *et al.*, 1992; Shimomura *et al.*, 2004). Other possibilities relate to interactions between Noc2 and the priming factor Munc13, and the cytoskeletal protein Zyxin. These have been identified by in vitro binding experiments and yeast 2-hybrid experiments respectively (Kotake *et al.*, 1997; Cheviet *et al.*, 2004a).

4.3.4 Munc18-1

The major findings reported here with respect to Munc18-1 function, were those showing that overexpression of the Munc18-1(E466K) mutant caused a redistribution of EGFP-Rab3A or EGFP-Rab27A-labeled secretory granules to the periphery of cells (Fig.4.7, F-J, Fig.4.8, A-E). It may be inferred that this effect is related to the enhanced secretion seen where this mutant is expressed in adrenal chromaffin or PC12 cells (Ciufo *et al.*, 2005; Graham *et al.*, 2008). In support of this inference, overexpression of the Munc18-1(E466K, R39C) double mutant was unable to produce either effect (Fig.4.7, F-J, Fig.4.8, A-E)(Graham *et al.*, 2008). It might also be suggested that both effects are linked to the increased binding of Munc18-1(E466K) to Rab3A from bovine brain lysate(Graham *et*

al., 2008). The E466 residue is exposed in the characterised structures of rat Munc18-1 and squid Sec1, consistent with its possible direct involvement in protein-protein interactions (Bracher *et al.*, 2000; Misura *et al.*, 2000). Further, peptides corresponding to the E466-containing domain III loop region of Munc18 are found to inhibit neurotransmitter release at the squid giant synapse, and plasma membrane translocation of Glut4 vesicles in adipocytes, consistent with a conserved functional role for this region (Dresbach *et al.*, 1998; Thurmond *et al.*, 2000).

Despite the coincident findings described above, previous reports mean that it may at first seem counterintuitive that the E466K mutation should enhance docking/tethering and secretion by promoting a Munc18-1-Rab3A interaction. The mutation is based on a mutation in the yeast SM protein Sly1p that suppresses the effects of deletion of yeast Rab protein Ypt1 (Dascher *et al.*, 1991; Ossig *et al.*, 1991): how could this effect be explained in terms of altered Rab-binding characteristics? Recent work characterising the Sly1p mutant suggests an answer to this question. It was reported that expression of the mutant allowed another Rab protein, Ypt6p, to substitute for Ypt1p in its role in ER-Golgi trafficking (Ballew *et al.*, 2005). The authors of this study suggested that mutant Sly1p bypassed a requirement for Ypt1p in membrane fusion, while Ypt6p was able to substitute in a role pre-fusion (Ballew *et al.*, 2005). However, a simpler explanation for their findings is that a putative requirement for a direct Sly1p-Ypt1p interaction was substituted by a Sly1p-Ypt6p interaction where Sly1p was mutated. Thus, the data from yeast may argue that the

E466K mutation influences the Munc18-1-Rab3A interaction directly, and may indicate that SM protein-Rab protein interactions are functionally significant in multiple membrane trafficking steps, and conserved through evolution.

Whatever the specific effect of the E466K mutation on protein-protein interactions, it is important to consider the mechanism by which enhanced binding of Rab3A to Munc18-1 might affect exocytosis. The function of Munc18-1 in exocytosis has long been the subject of great interest. It is known to have an essential role in neurotransmitter release, as no neurotransmission occurs in Munc18-1 -/- mice (Verhage *et al.*, 2000), and it is thought that this role is exerted through its interactions with the SNARE protein Syntaxin1A (Burgoyne and Morgan, 2007). Several modes of Munc18-1-Syntaxin1A binding have now been identified. Initially, Munc18-1 was shown to bind to and stabilise a 'closed' conformation of Syntaxin1A, unable to interact with other SNARE proteins, and therefore unable to mediate membrane fusion (Dulubova *et al.*, 1999). More recently however, a positive effect of Munc18-1 in in vitro membrane fusion assays has been found to be dependent on its binding to an N-terminal region of the Syntaxin (Shen *et al.*, 2007). This interaction can occur when Syntaxin1A forms part of an assembled SNARE complex, and can occur in vivo (Dulubova *et al.*, 2007; Rickman *et al.*, 2007). Binding of Munc18-1 to the N-terminal domain of Syntaxin1A is likely to occur in a manner analogous to the binding of SM proteins Sly1p and Vps45p to their appropriate Syntaxins (Bracher and Weissenhorn, 2002; Dulubova *et al.*,

2002). However, where Munc18-1 binds an assembled SNARE complex, binding to the N-terminal region of Syntaxin1A may be combined with additional interactions including interaction with the v-SNARE VAMP2, reflecting an additional binding mode. Binding of Munc18-1 to VAMP2 has been found critical for its stimulation of in vitro membrane fusion (Shen *et al.*, 2007), and it is possible that Munc18-1 interacts with assembled SNARE complexes in a manner analogous to that of Sec1 (Carr *et al.*, 1999; Scott *et al.*, 2004).

The binding of Munc18-1 to 'closed' Syntaxin1A has been previously suggested to stabilise the Syntaxin, and prevent its inappropriate incorporation into SNARE complexes (Rowe *et al.*, 1999). Such a role may be important while the protein is trafficked to the plasma membrane (Rickman *et al.*, 2007), and as discussed, where 'inert' granules are docked/tethered by a mechanism involving Granuphilin (Fukuda *et al.*, 2005). However, it was found in this laboratory that the Munc18-1 R39C mutation, which selectively reduces the binding of Munc18-1 to 'closed' Syntaxin, was able to abolish the positive functional effects of the E466K mutation in terms of granule distribution (Fig.4.7, I-J, Fig.4.8, D-E) and secretion (Graham *et al.*, 2008). This was not due to an effect on the Rab3A interaction, as enhanced binding to this protein was retained in pull-down experiments using the double mutant (Graham *et al.*, 2008). Therefore, these findings suggest that 'closed' Syntaxin may also represent a 'conformational precursor' early in a system of sequential interactions driving SNARE engagement and leading docking or fusion.

Different Munc18-1-Syntaxin binding modes may operate at different points in this system, while it is likely that Munc18-1(E466K) overexpression – increased Rab3A-Munc18-1 interaction - potentiates the system at a point upstream of a transition between 'closed' and 'open' Syntaxin. This is supported by findings showing that microinjection of GTPase-deficient Rab3A into Aplysia neurones can delay the onset of inhibition of neurotransmitter release produced by Clostridial neurotoxins that cleave the SNARE proteins VAMP and SNAP-25 where these proteins are isolated, but not where they form parts of assembled SNARE complexes (Johannes *et al.*, 1996).

If Rab3A-Munc18-1 interaction can potentiate SNARE engagement, the nature of its regulation should be discussed. In order to discuss this, it is necessary to consider the regulation of exocytotic machinery in endocrine and neuroendocrine cells as a whole. Regulated exocytosis in these cells differs from other membrane trafficking pathways, in that it is important for cells to be predictably responsive to a range of temporally unpredictable stimuli. Thus, cells maintain releasable and readily releasable pools of secretory granules, through a number of different mechanisms (Burgoyne and Morgan, 2003). While granules are continually synthesised, it is thought that newly synthesised secretory granules are more fusion competent than older granules (Duncan *et al.*, 2003). Immature granules are transported to the periphery of cells for maturation, while such granules may still undergo exocytosis (Tooze *et al.*, 1991; Rudolf *et al.*, 2001). Mature granules may remain at the periphery of cells and may

become tethered or docked at the plasma membrane, while over time it is thought that these granules progressively lose mobility and fusion competence and are finally displaced and sequestered in the cell body (Rudolf *et al.*, 2001; Duncan *et al.*, 2003). However, it is not only tethered or docked granules that can contribute to exocytotic responses. Where cells are stimulated, undocked granules may be mobilised towards the plasma membrane and these granules may be efficiently exocytosed (Han, 1999). Therefore, mechanisms by which granules lose fusion competence with time are likely to be complimented by mechanisms that promote granule mobilisation, tethering, docking, priming and fusion under stimulation, and that thereby mediate exocytosis of both docked and undocked granules. In this context, it is likely that there is a cellular requirement to control the degree to which the Rab3A-Munc18-1 interaction is promoted or repressed under many different circumstances.

Because Munc18-1(E466K) overexpression causes peripheral redistribution of relatively newly synthesised EGFP-Rab3A/EGFP-Rab27A-labeled secretory granules (Fig.4.7, F-J, Fig.4.8, A-E), but not all granules (Fig.4.8, F-G), it is likely that mechanisms limiting tethering/docking of older granules still function under these conditions. While Munc18-1(E466K) may preferentially increase tethering/docking of more fusion competent granules, its overexpression in adrenal chromaffin cells led to an increased frequency of exocytotic events compared to controls, and this increase was sustained over three minutes of stimulation (Ciufo *et al.*, 2005). This suggests that mechanisms modulating fusion competence of

more fusion competent granules per se may be dysregulated when the mutant is expressed. In PC12 cells, siRNA knockdown of Rab3A expression leads to a ~40% reduction in exocytotic responses compared to controls (Tsuboi and Fukuda, 2006a), while in neurones lacking Rab3A, the release probability of a subset of synaptic vesicles of the readily releasable pool is reduced (Schluter *et al.*, 2004). In the previous chapter, it was speculated that differential interactions between synaptic vesicles, and the cycling pool of Rab3A, according to vesicle age, might underlie corresponding differences in fusion competence. The data presented in this chapter might support this speculation, since it indicates that fusion competence may be determined in part, by a complex system coordinating Munc18-1 function via Rab3A.

Chapter 5:

RabGAPs in neuroendocrine exocytosis

5.1 Introduction

Successive targeting of GDI-associated Rab protein to a specific membrane compartment, GEF-catalysed exchange of bound GDP for GTP, association between the Rab protein and its effectors, and in some cases the formation of Rab domains, have been suggested to constitute the Rab activation pathway (Takai *et al.*, 2001). Successive RabGAP-promoted Rab GTP-hydrolysis and GDI-mediated Rab membrane extraction have been suggested to constitute their inactivation pathway (Takai *et al.*, 2001). While Rab activation and inactivation may be grossly associated with the progression and termination of a given membrane trafficking step respectively (Sudhof, 1997; Takai *et al.*, 2001), the work carried out in this study has suggested that continual dynamic transitions of Rab proteins between different activation states may also define their influence on cellular events (Handley *et al.*, 2007). Therefore, characterisation of the protein regulators of such transitions may be of critical importance in the future investigation of Rab protein function. Given the limitations of time, the final portion of this study was focused on one class of regulatory protein: the RabGAP.

RabGAPs interact with GTP-bound Rab proteins and increase their hydrolytic activity. The majority of identified RabGAPs are TBC (Tre-2, Bub-2, Cdc16) domain-containing proteins, and are thought to accelerate Rab GTP-hydrolysis by a common mechanism (Pan *et al.*, 2006). While GAPs for each Rab protein have not yet been identified, sequence

analysis has identified >35 TBC domain-containing proteins, and it is thought likely that each of these putative GAPs acts on a specific Rab subtype or isoform (Haas *et al.*, 2005; Itoh *et al.*, 2006). Since this study initially focused on the distribution, dynamics and function of Rab3A and Rab27A, and their effectors in PC12 cells, work was directed towards characterisation of candidate RabGAPs specific for these Rab isoforms.

5.2 Results

5.2.1 Characterisation of proteins reported to function as GTPase-activating proteins for Rab3A and Rab27A

RabGAPs interact with GTP-bound Rab proteins and potentiate GTP-hydrolysis (Takai *et al.*, 2001; Pan *et al.*, 2006). As GTP-bound Rab proteins are generally considered 'active' while GDP-bound Rab proteins may be vulnerable to GDI-mediated membrane extraction, RabGAP activity is important for the termination of discrete membrane trafficking steps. Where a transport vesicle fuses with its target organelle, for example, GTP-hydrolysis by the Rab protein regulating the process, and the subsequent extraction of the GDP-bound Rab is required to prevent accumulation of this protein (Novick and Brennwald, 1993; Simons and Zerial, 1993; Sudhof, 1997; Takai *et al.*, 2001). However, it is possible that GAP activity may also be required in order to maintain the size of a vesicle-associated pool of a Rab protein, or to maintain rates of Rab GTP-hydrolysis and reassociation (see previous chapters). Evidence supporting this suggestion includes findings that overexpression of specific RabGAPs can dramatically reduce the membrane-association of their target Rabs. For example, RabGAP5 overexpression is associated with dispersal of Rab5 in HeLa cells (Haas *et al.*, 2005).

The protein p130 Rab3GAP is suggested to potentiate Rab3A GTP-hydrolysis (Fukui *et al.*, 1997; Oishi *et al.*, 1998; Sakane *et al.*, 2006), and this is suggested to be coupled to exocytotic signalling and/or the fusion of

synaptic vesicles with the plasma membrane (Sudhof, 1997). Because RabGAP activity might also underlie extraction of granular Rab3A under resting conditions in PC12 cells, the effects of p130 Rab3GAP-myc overexpression on EGFP-Rab3A-granule association were analysed. In order to increase the likelihood of observing an effect, cells imaged were those with relatively strong anti-myc staining, and relatively weak EGFP-Rab3A fluorescence. To maximise the number of cells fitting this description, transfection mixes were made-up to contain 0.75μg of the GAP-encoding plasmid, and 0.25μg of the EGFP-Rab3A-encoding plasmid per transfection. Consistently, EGFP-Rab3A showed a similar localisation in these cotransfected cells to that previously shown where it was transfected alone; the protein was localised to punctae distributed throughout the cytosol and at the periphery of cells (Fig.5.1, A). p130 Rab3GAP-myc was also associated with punctae in some cells (Fig.5.1, B), while in others, it had a more diffuse distribution (Fig.5.1, E). p130 Rab3GAP-myc-labeled punctae did not colocalise with those labeled with EGFP-Rab3A (Fig.5.1, A-C). Similarly, Rab3GAP-myc did not colocalise with, or affect the punctuate distribution of, EGFP-Rab27A (Fig.5.1, D-F).

While overexpression of p130 Rab3GAP-myc produced no observable effects on EGFP-Rab3A or EGFP-Rab27A localisation in resting cells, it was possible that it might produce functional effects on exocytosis. In order to identify such effects, the protein was expressed in an hGH cotransfection assay. However, no change in ATP-stimulated hGH secretion was identified (Fig.5.1, G). Similar findings have been reported

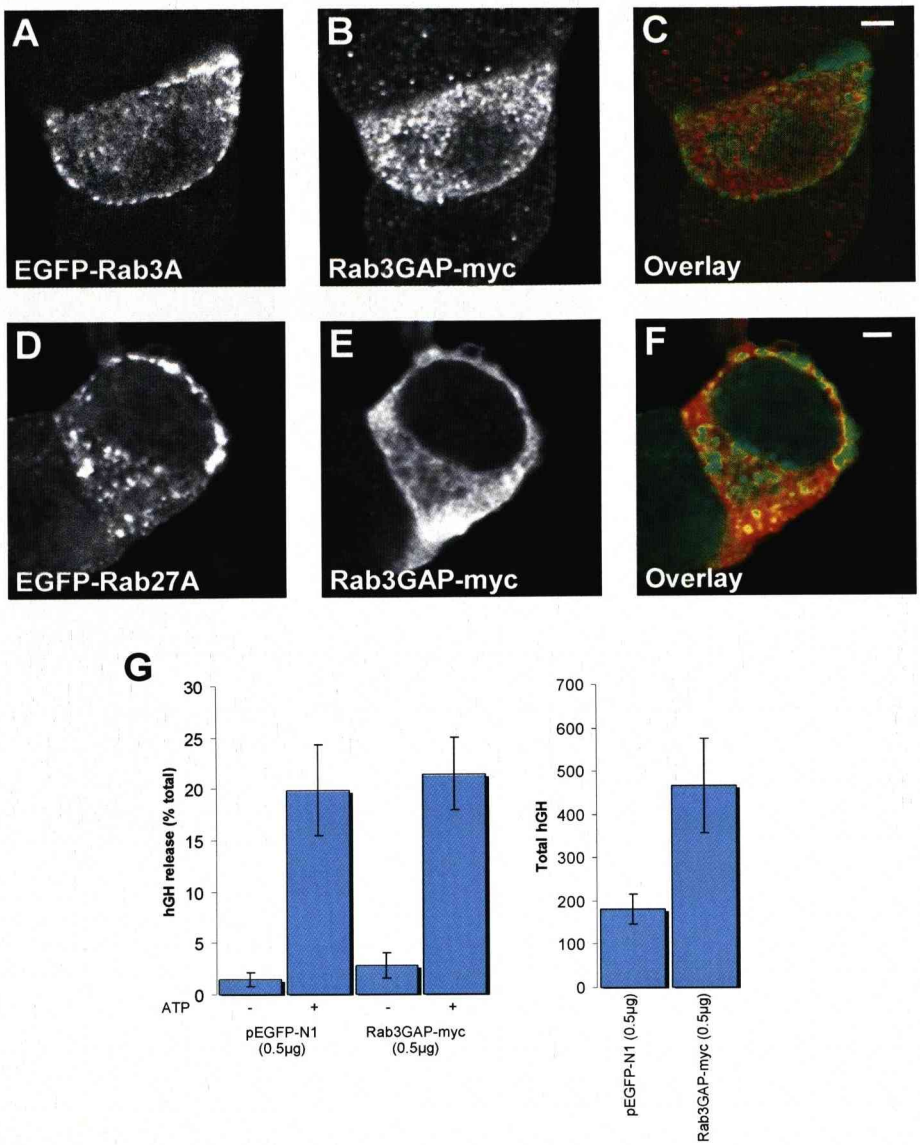

Figure 5.1. p130 Rab3GAP-myc localisation and effects in PC12 cells. (A-F) Cells were cotransfected with p130 Rab3GAP-myc, and either EGFP-Rab3A (A-C), or EGFP-Rab27A (D-F), fixed 18 hours following transfection, and then immunostained with anti-myc. Each overlay image on the right of the figure is a composite of green from the left image and red from the centre image. Areas of overlap appear in yellow.. Bars, 4µm. (G) Secretion of exogenous human growth hormone (hGH) from cells cotransfected with plasmids encoding EGFP or p130 Rab3GAP-myc was assayed. Stimulated cells were exposed to 300 µM ATP, hGH release over 15 minutes was assayed and expressed as a percentage of total hGH (shown to the right of the figure). Data are expressed as mean ± s.e.m. (n=6)

previously (Oishi *et al.*, 1998), and may indicate a minimal role for p130 Rab3GAP upstream of the exocytotic fusion event.

The proteins TBC1D10A and TBC1D10B have recently been reported to exert GAP activity towards Rab27A, both in vitro and in vivo (Itoh and Fukuda, 2006). On this basis, the localisation and effects of myc-tagged forms of these proteins were explored. When TBC1D10A-myc was coexpressed with either EGFP-Rab27A (Fig.5.2, A-F), or EGFP-Rab3A (Fig.5.2, G-L), in PC12 cells, anti-myc staining was concentrated at the periphery of cells (Fig.5.2, B, E, H, K). This staining pattern would be consistent with the protein's localisation to cortical F-actin, given its similarity to that produced previously when cells were stained with phalloidin (see Fig.4.9, A). When cells coexpressing EGFP-Rab27A were imaged, EGFP-Rab27A fluorescence was largely cytosolic in ~50% of cases (Fig.5.2, A-C). In the remainder of cells, this fluorescence was punctate, and characteristic of typical EGFP-Rab27A localisation (Fig.5.2, D-F). In these cells, EGFP-Rab27A-labeled punctae were not excluded from cortical areas of high anti-myc fluorescence.

The capacity of TBC1D10A-myc to promote dispersal of EGFP-Rab27A in a proportion of cotransfected cells might be consistent with direct GAP activity. However, where corresponding experiments were carried out in cells cotransfected with EGFP-Rab3A, this protein was largely cytosolic in a similar proportion of cells, a possible indication that any GAP activity was non-specific (Fig.5.2, G-L). Despite this, the functional effects of TBC1D10A-myc overexpression were explored. Since Rab27A may act through multiple effectors in PC12 cells, the effects of any GAP-mediated increase in its rate of its GTP-hydrolysis may be difficult to predict.

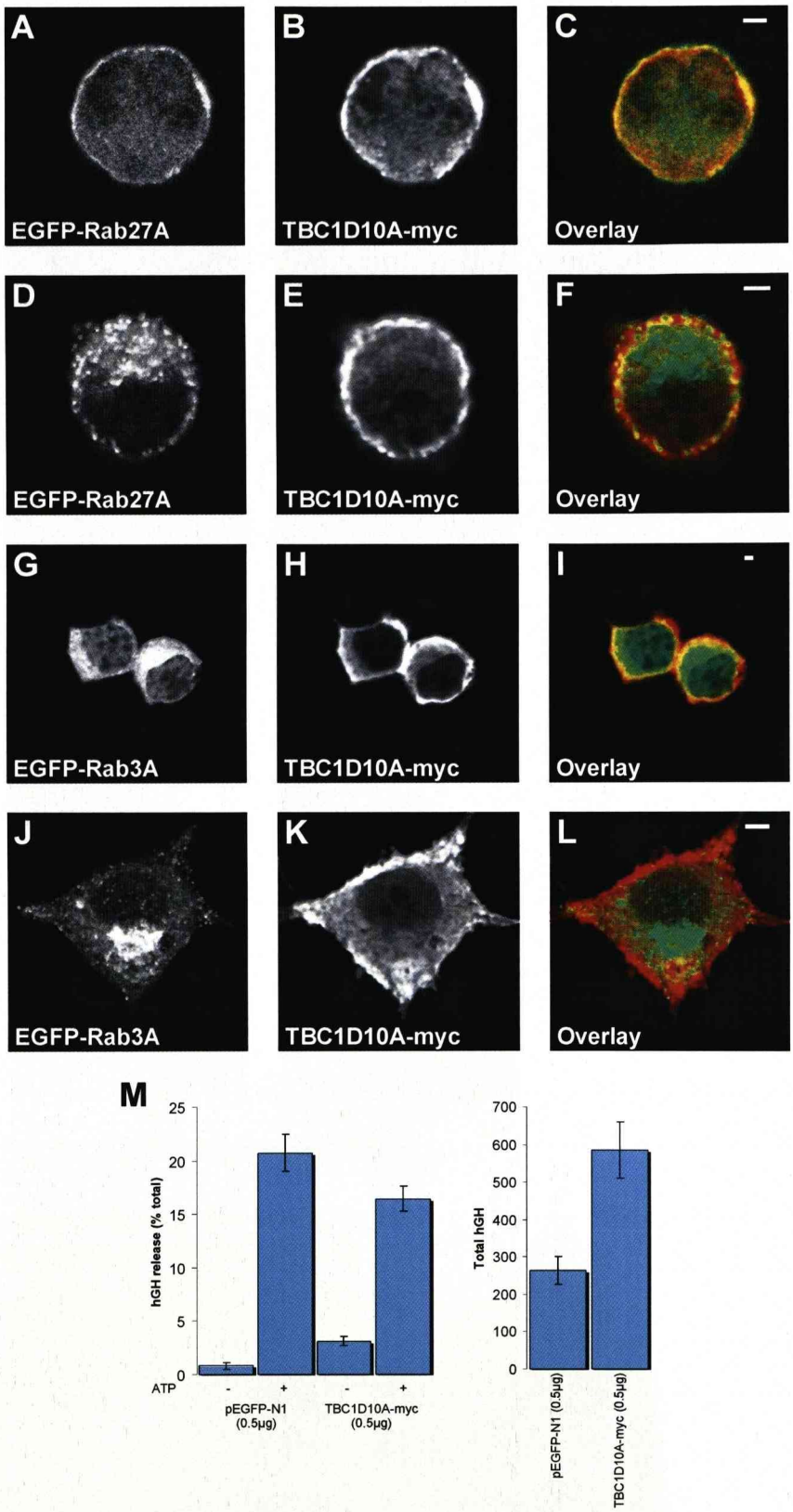

Figure 5.2. TBC1D10A-myc localisation and effects in PC12 cells. (A-L) Cells were cotransfected with TBC1D10A-myc, and either EGFP-Rab27A (A-F), or EGFP-Rab3A (G-L), fixed 18 hours following transfection, and then immunostained with anti-myc. Each overlay image on the right of the figure is a composite of green from the left image and red from the centre image. Areas of overlap appear in yellow. Bars, 4µm. [contd. on next page]

Figure 5.2 contd. (M) Secretion of exogenous human growth hormone (hGH) from cells cotransfected with plasmids encoding EGFP or TBC1D10A-myc was assayed. Stimulated cells were exposed to 300 μM ATP, hGH release over 15 minutes was assayed and expressed as a percentage of total hGH (shown to the right of the figure). Data are expressed as mean ± s.e.m. (n=6)

However, it is possible that the resulting reduction in GTP-Rab27A levels, and in the protein's granule-association, could be expected to mirror the inhibitory effects of a partial siRNA knockdown (Waselle *et al.*, 2003; Tsuboi and Fukuda, 2006a). When the effects of TBC1D10A-myc overexpression were assessed in an hGH cotransfection assay, it was found to cause a small reduction in ATP-stimulated hGH secretion (Fig.5.2, M).

TBC1D10B-myc overexpression produced similar effects on the distribution of the coexpressed EGFP-tagged Rab3A and Rab27A proteins to those produced by TBC1D10A-myc (Fig.5.3, A-L). In ~50% of the cells imaged, the EGFP-Rab fluorescence was diffuse, while in the remainder, it was characteristically punctate. Again these results may indicate that the putative GAP exerts a non-specific GAP activity. Like TBC1D10A-myc, TBC1D10B-myc localised to the periphery of cells (Fig.5.3, B, E, H, K). However, in some cells it was seen to label large vesicular/vacuolar structures (Fig.5.3, E, K). Given the size of these structures, and in some cases, their visible exclusion of EGFP-Rab-labeled punctae from regions of the cytosol (Fig.5.3, E), it is very likely that they constitute a dramatic change in normal cellular physiology, perhaps resulting from dysregulation of some aspect of membrane trafficking. This dysregulation, rather than a specific effect of the protein on Rab27A function, may underlie the large

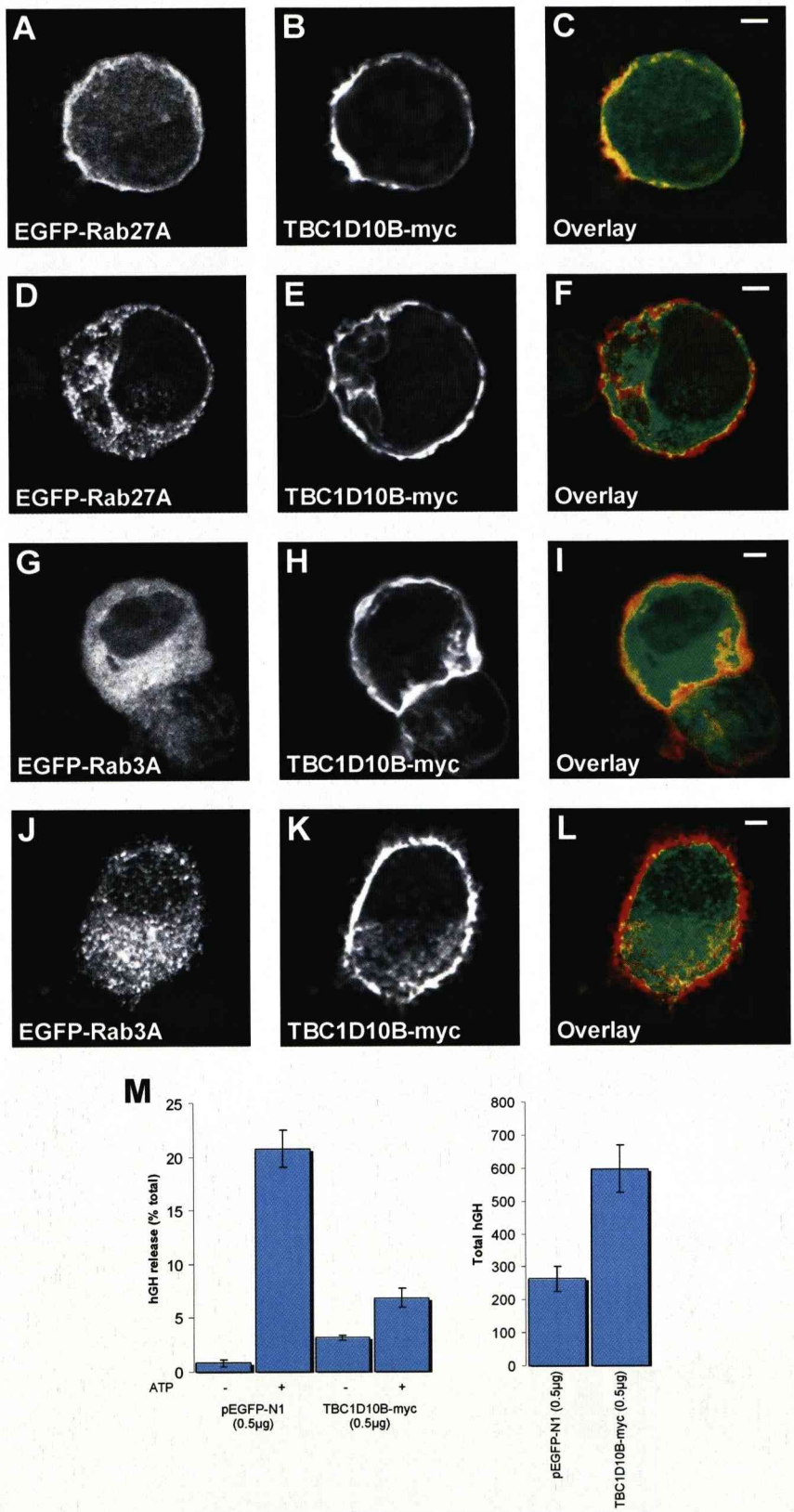

Figure 5.3. TBC1D10B-myc localisation and effects in PC12 cells. (A-L) Cells were cotransfected with TBC1D10B-myc, and either EGFP-Rab27A (A-F), or EGFP-Rab3A (G-L), fixed 18 hours following transfection, and then immunostained with anti-myc. Each overlay image on the right of the figure is a composite of green from the left image and red from the centre image. Areas of overlap appear in yellow. Bars, 4µm. [contd. on next page]

Figure 5.3 contd. (M) Secretion of exogenous human growth hormone (hGH) from cells cotransfected with plasmids encoding EGFP or TBC1D10B-myc was assayed. Stimulated cells were exposed to 300 μM ATP, hGH release over 15 minutes was assayed and expressed as a percentage of total hGH (shown to the right of the figure). Data are expressed as mean ± s.e.m. (n=6)

reduction in ATP-stimulated exogenous hGH secretion produced where TBC1D10B-myc was coexpressed (Fig.5.3, M).

5.2.2 Screening of putative RabGAPs to identify potential activity towards Rab3A and Rab27A

In the experiments described above, it was found that p130 Rab3GAP-myc overexpression produced no effect on the localisation of EGFP-Rab3A in cotransfected cells or on secretory responses as gauged by hGH cotransfection assays. It was also found that overexpression of myc-tagged forms of the putative Rab27 GAPs TBC1D10A and TBC1D10B was associated with variable redistribution of both EGFP-Rab27A and EGFP-Rab3A, and that TBC1D10B-myc overexpression was associated with changes in cellular morphology. Given these data, it is possible that p130 RabGAP does not function as a GAP for Rab3A in PC12 cells, or does so only following the exocytotic event. Further, it is possible that TBC1D10A and TBC1D10B show non-specific GAP activity, and are not physiological regulators of Rab27A function. It was therefore decided to screen an additional 34 putative RabGAPs to identify alternative candidate Rab3A and Rab27A-specific GAPs.

Following on from the previous work, initial experiments were directed towards identification of GAPs capable of altering the localisation of the

EGFP-labeled Rab proteins on their overexpression. cDNAs encoding the putative RabGAPs were previously subcloned into a modified form of the pcDNA3.1(+) vector (Invitrogen, UK) encoding a myc-tag, and the resulting plasmids were kindly provided for this study by F Barr (School of Cancer Studies, University of Liverpool). As before, cells were cotransfected using 0.75μg of each GAP-encoding construct, and 0.25μg of either EGFP-Rab3A or EGFP-Rab27A, in order to maximise the numbers of cells coexpressing relatively high levels of GAP, and moderate levels of Rab protein. Because each recombinant GAP carried the same epitope tag, and was labeled in cells using the same antibody and according to the same protocol, it was possible to identify cells expressing comparable levels of these proteins for analysis, despite some differences in their expression efficiency.

Multiple cells expressing each combination of GAP and Rab protein were imaged, but given the volume of data that was produced in the screening process, it is presented here in tabulated form (Table 5.1). The effects of each GAP on the localisation of coexpressed Rab protein were scored qualitatively, according to the proportion of cells in which the specified Rab appeared uncharacteristically diffuse. In the table, '+', '++' and '+++' indicate that the Rab was largely diffuse in ~25%, ~50% or >75% of the cells imaged respectively, and the localisation of each GAP protein is characterised as either cytosolic, vesicular, perinuclear, or peripheral (see Fig.5.4). Where a particular GAP seemed to adopt different localisations in different cells, it usually appeared diffuse in some, but either vesicular or

GAP	Localisation	Rab3A	Rab27A	Probable Target
RabGAP-5	Vesicles/Perinuclear			Rab5A-C (Haas *et al.*, 2005; Fuchs *et al.*, 2007)
Paris1	Vesicles/Cytosolic		+	
Rab3GAP	Vesicles/Cytosolic			Rab3 (Fukui *et al.*, 1997)
RUTBC2	Vesicles		++	
GAPCenA	Vesicles/Perinuclear			Rab4 (Fuchs *et al.*, 2007)
Gyp6	Vesicles			
USP6	Peripheral	+		
TBC1D22B	Vesicles	+	+	Rab33 (Pan *et al.*, 2006)
TBC1D1	Vesicles		+	
TBC1D16	Vesicles			
EVI5	SGs	+	++	Rab35 (Fuchs *et al.*, 2007)
RN-tre	Peripheral	+	+	Rab41 (Haas *et al.*, 2005; Fuchs *et al.*, 2007)
RUTBC1	SGs	+++		
TBC1D3	Peripheral		++	
TBC1D7	Cytosolic	+		Rab17 (Yoshimura *et al.*, 2007)
TBC1D10A	Peripheral	++	++	
TBC1D10B	Peripheral	++	++	Rab22a/31 (Fuchs *et al.*, 2007)
TBC1D10C	Cytosolic	+	+	
TBC1D12	Vesicles		+	
TBC1D13	Vesicles	+		
TBC1D14	Vesicles		+	
TBC1D15	Vesicles		+	Rab7 (Zhang *et al.*, 2005)
TBC1D17	Vesicles/Cytosolic		+	Rab21 (Fuchs *et al.*, 2007)
TBC1D18	Vesicles	+		
TBC1D19	Vesicles			
TBC1D20	Vesicles	++		Rab1/2 (Haas *et al.*, 2007)
TBC1D21	Vesicles			
Gyp1	Vesicles		+	Rab33 (Pan *et al.*, 2006)
AK074305	Vesicles		++	
AAH_33712	Vesicles/Cytosolic			
KIAA0882	Vesicles		+	
NM_018309	Vesicles/Perinuclear		+	
Evi5-like	Vesicles			Rab23 (Yoshimura *et al.*, 2007)
XM_037557	Vesicles/Perinuclear		+	Rab8A (Yoshimura *et al.*, 2007)
XM_370928	Vesicles/Cytosolic			
NM_198868	Vesicles			
TBC1D4	Vesicles			

Table 5.1. Screening putative RabGAPs. Cells were transfected to coexpress a myc-tagged version of each RabGAP listed, and either EGFP-Rab3A, or EGFP-Rab27A, fixed 18 hours following transfection, and then immunostained with anti-myc. RabGAP localisation was subjectively characterised according to anti-myc staining. For each condition, cells stained strongly with anti-myc, and weakly with EGFP-Rab, were selected and scored for EGFP-Rab dispersal. '+' indicates dispersal in >25% cells, '++' ~50%, and '+++' 75%. A minimum of 10 cells were scored where '++' and '+++' dispersal is indicated.

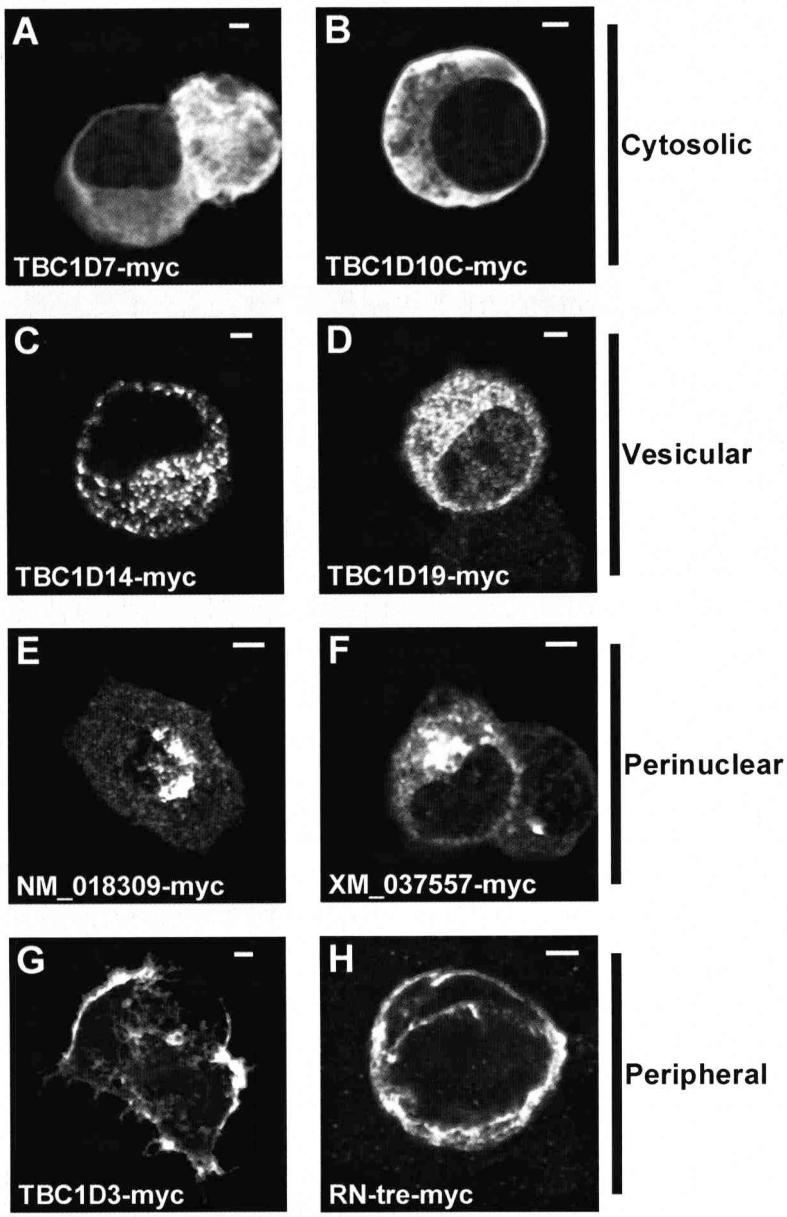

Figure 5.4. Subjective characterisation of RabGAP localisation (A-H) Imaged cells were fixed 18 hours after transfection with plasmids encoding the RabGAPs indicated, and immunostained with anti-myc. Localisations were characterised as indicated. Bars, 4μm.

perinuclear in others. Since a large diffuse component to GAP fluorescence might mask a more restricted localisation underlying it, the appearance of a cytosolic localisation was discounted in such cases.

Broadly, the results of the screen appear to indicate that a relatively high proportion of the RabGAPs tested can exert some effect on the localisation of either or both of the EGFP-Rab proteins, and that more can affect EGFP-Rab27A than can affect EGFP-Rab3A. However, caution should be taken before drawing these conclusions, as effects were typically noted in only a small proportion of cells and may represent false positives. Where EGFP-Rab protein fluorescence was diffuse in 50% or more of imaged cells, this is much more likely to reflect an effect of the coexpressed GAP. If only these cases are considered, the screen identified two RabGAPs associated with dispersal of EGFP-Rab3A, and four associated with dispersal of EGFP-Rab27A.

RabGAPs associated with dispersal of EGFP-Rab3A were TBC1D20, and RUTBC1, both of which showed a vesicular localisation. The effects of TBC1D20-myc overexpression on EGFP-Rab3A were more variable than those of RUTBC1-myc, and the available information suggests that they were indirect. TBC1D20 is reported to function as a GAP for Rab1, and its overexpression is found to disrupt trafficking from the ER, and to cause loss of the Golgi complex (Haas *et al.*, 2007). Therefore, where it is overexpressed, EGFP-Rab3A may be mislocalised as a result of reduced or aberrant secretory granule synthesis.

The effects of RUTBC1-myc overexpression were striking. Expression of this protein was associated with diffuse EGFP-Rab3A fluorescence in almost all of the coexpressing cells imaged, while no such effect on

coexpressed EGFP-Rab27A was apparent (Fig.5.5, A-F). Further analysis showed that in fact, punctae labeled with RUTBC1-myc colocalised with those labeled with EGFP-Rab27A, suggesting that the protein is targeted to secretory granules (Fig.5.5, D-F). These results are significant, because while its dispersal of EGFP-Rab3A suggests only possible Rab3GAP activity, the protein's localisation on granules may support the suggestion that such activity could be physiologically relevant.

As a candidate GAP for Rab3A, it was possible that RUTBC1 overexpression might affect PC12 cell secretory responses by reducing the quantity and/or activity of endogenous Rab3A on secretory granules. However, when the effects of RUTBC1-myc overexpression were quantified in an hGH-cotransfection assay, ATP-stimulated secretion was unaffected (Fig.5.5, G). Whereas the siRNA knockdown of Rab3A in PC12 cells is found to reduce PC12 cell secretory responses by ~40%, its overexpression can produce even more dramatic reduction in secretion (Chung *et al.*, 1999; Tsuboi and Fukuda, 2006a). Because reduced secretion resulting from Rab3A overexpression depends on the protein's membrane-association (Johannes *et al.*, 1996), while immunocytochemical data suggested that RUTBC1-myc overexpression might reduce this, the effects of cooverexpression of EGFP-Rab3A and RUTBC1-myc were investigated. Transfection of cells with 0.25μg of a plasmid encoding EFGP-Rab3A was found to cause a ~50% reduction in ATP-stimulated secretion as compared to controls. When cells were simultaneously transfected with 0.75μg of a plasmid encoding RUTBC1-myc, no reduction

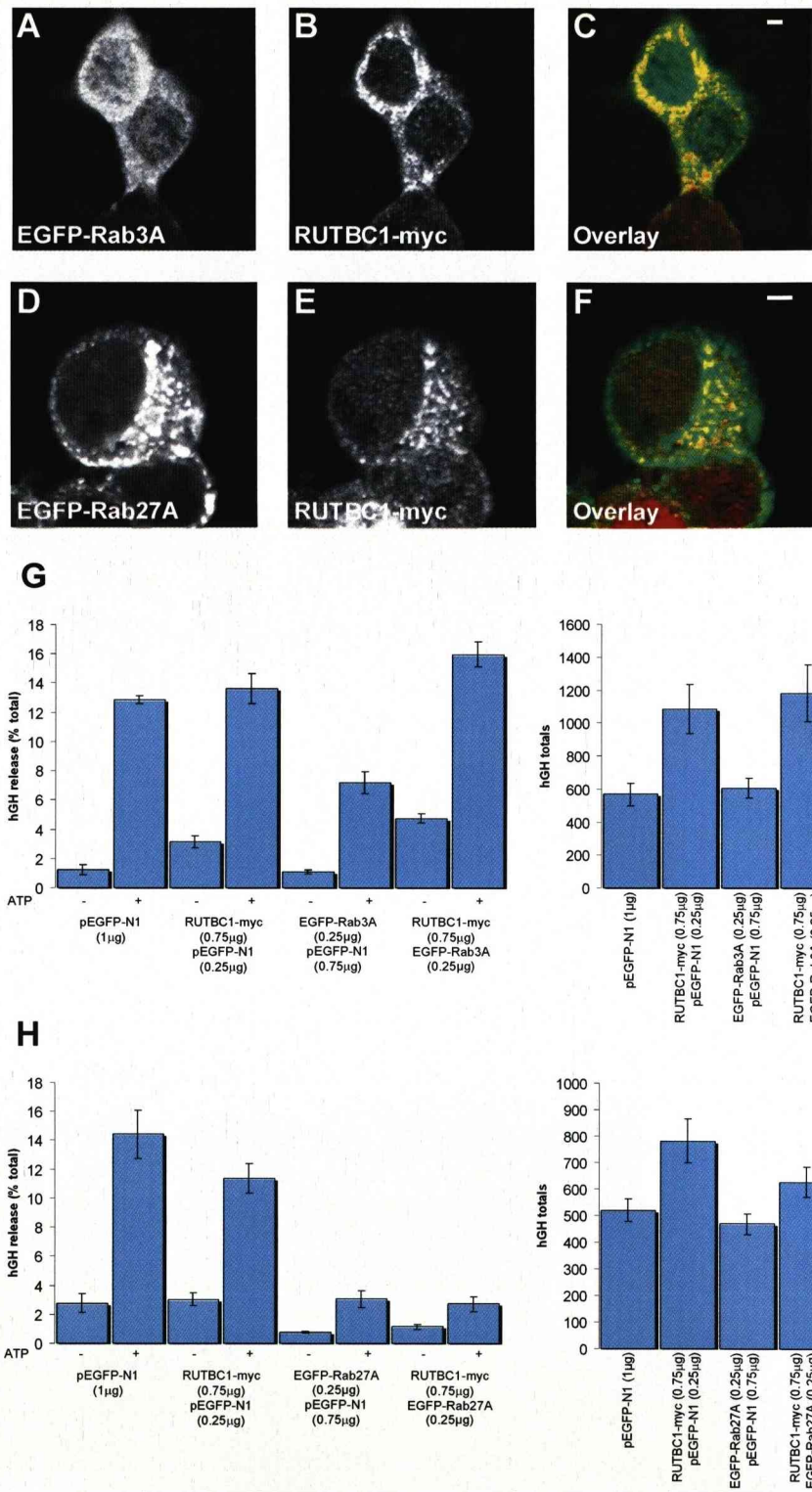

Figure 5.5. RUTBC1-myc localisation and effects in PC12 cells. (A-F) Cells were cotransfected with RUTBC1-myc, and either EGFP-Rab3A (A-C), or EGFP-Rab27A (D-F), fixed 18 hours following transfection, and then immunostained with anti-myc. Each overlay image on the right of the figure is a composite of green from the left image and red from the centre image. Areas of overlap appear in yellow. Bars, 4µm. (G-H) Secretion of exogenous human growth hormone (hGH) from cells cotransfected with the indicated plasmids was assayed 48 hours following transfection. Stimulated cells were exposed to 300 µM ATP, hGH release over 15 minutes was assayed and expressed as a percentage of total hGH (shown to the right of the figure). Data are expressed as mean ± s.e.m. (In G, n=6)(In H, n=3)

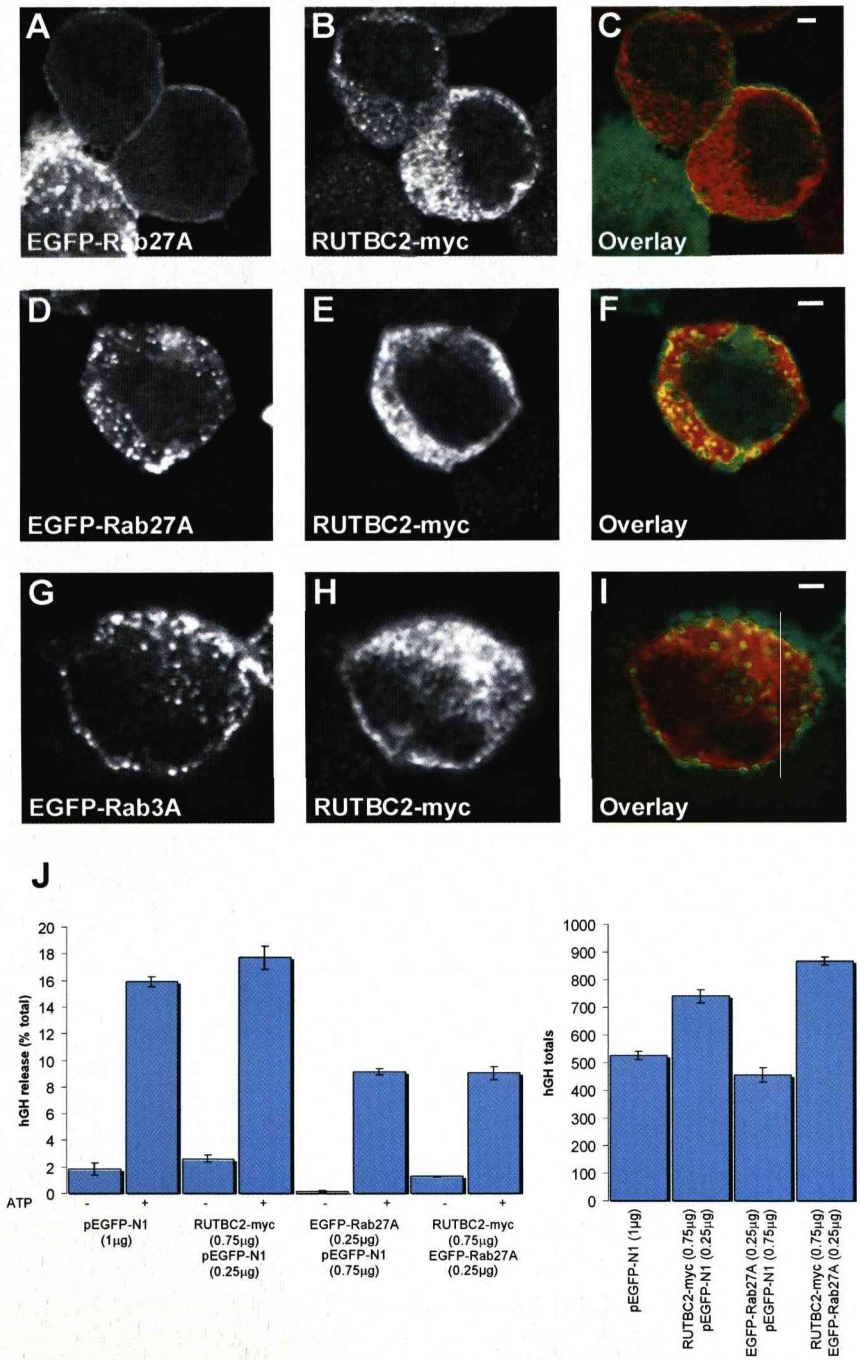

Figure 5.6. RUTBC2-myc localisation and effects in PC12 cells. (A-I) Cells were cotransfected with RUTBC2-myc, and either EGFP-Rab27A (A-F), or EGFP-Rab3A (G-I), fixed 18 hours following transfection, and then immunostained with anti-myc. Each overlay image on the right of the figure is a composite of green from the left image and red from the centre image. Areas of overlap appear in yellow. Bars, 4μm. (J) Secretion of exogenous human growth hormone (hGH) from cells cotransfected with the indicated plasmids was assayed 48 hours following transfection. Stimulated cells were exposed to 300 μM ATP, hGH release over 15 minutes was assayed and expressed as a percentage of total hGH (shown to the right of the figure). Data are expressed as mean ± s.e.m. (n=3)

was seen (Fig.5.5, G). This data indicates that RUTBC1 may efficiently

promote membrane-extraction of supraphysiological Rab3A. In support of the suggestion that RUTBC1 exerts Rab3-specific effects, RUTBC1-myc overexpression was not found to suppress the inhibitory effects of EGFP-Rab27 on secretion (Fig.5.5, H).

RabGAPs associated with the dispersal of EGFP-Rab27A were RUTBC2, Evi5, TBC1D3 and AK_074305. Rab27 and Rab3 isoforms are more closely related to one another in terms of phylogeny than they are to any other Rabs (Pereira-Leal and Seabra, 2000), RUTBC2 and RUTBC1 are similarly related (Itoh *et al.*, 2006), and so the possibility that RUTBC2 may function as a GAP for Rab27A was addressed. The effects of RUTBC2-myc on cooverexpressed EGFP-Rab27A were dramatic in some cells (Fig.5.6, A-C). However, they were also more variable than those of RUTBC1-myc on EGFP-Rab3A, with EGFP-Rab27A displaying a punctate distribution in ~50% of imaged cells (Fig.5.6, D-F). Additionally, unlike RUTBC1-myc, RUTBC2-myc did not appear to be concentrated on secretory granules, as it did not colocalise with punctae labeled with either EGFP-Rab27A, or EGFP-Rab3A (Fig.5.6, A-I). In hGH cotransfection assays, it was found that RUTBC2-myc overexpression did not suppress the inhibitory effects of EGFP-Rab27A on secretion (Fig.5.6, J).

5.3 Discussion

5.3.1 Rab3 GTPase-activating proteins

The atypical RabGAP, 'Rab3GAP', was originally isolated following multiple fractionations of a soluble synaptic fraction of rat brain that was found to promote Rab3A [γ-^{32}P]GTP-hydrolysis. Following sequence analysis of the major protein, p130, in the highly purified 'GAP' preparation, it was identified, cloned, found to possess Rab3 GAP activity in vitro, and named accordingly (Fukui *et al.*, 1997). Another protein present in the 'GAP' preparation, p150, was later characterised as a noncatalytic subunit of Rab3GAP (Nagano *et al.*, 1998). While it does not affect GAP activity in vitro, it may be important for GAP function in vivo since mutations in both proteins are associated with related genetic disorders characterised by ocular and neurodevelopmental defects; Warburg micro syndrome and Martsolf syndrome (Aligianis *et al.*, 2005; Aligianis *et al.*, 2006). Rab3GAP is structurally very different from other characterised GAPs, although it is thought to function in an analogous manner, and orthologues exist in several species (Clabecq *et al.*, 2000). Like other RabGAPs, Rab3GAP has relatively low affinity for its substrate GTP-Rab, but greater affinity where the Rab adopts a conformation thought to constitute an intermediate state associated with GTP hydrolysis, and an argenine residue in its catalytic site is critical for its GAP activity (Clabecq *et al.*, 2000).

Consistant with the crude inference that altered Rab3 regulation of neurotransmission in Warburg micro syndrome may result in the neurodevelopmental defects seen in this disorder, a number of studies have provided evidence that Rab3GAP is involved in Rab3 GTP-hydrolysis in vivo. The protein colocalises with Rab3 in synaptic terminals (Oishi *et al.*, 1998), and it is found that GTP-Rab3A accumulates in the brains of Rab3GAP knockout mice (Sakane *et al.*, 2006). Further, CA1 hippocampal neurones taken from these mice show enhanced short-term plasticity (STP) on repetitive stimulation, and enhanced paired-pulse facilitation (PPF), a nearly identical phenotype to that observed in GDIα knockout mice (Ishizaki *et al.*, 2000; Sakane *et al.*, 2006). This is significant, because both GAP-catalysed Rab3 GTP hydrolysis and GDIα-mediated GDP-Rab3 sequestration regulate the extraction of Rab3 from synaptic vesicles, and so the effects of both deletions might arise from parallel increases in vesicle-associated Rab3. In support of this suggestion, another report showed that relative STP and PPF were also enhanced when cellular levels of the Rab3 orthologue apRab3 were directly increased in Aplysia neurones through microinjection, though it should be noted that the net stimulus-evoked ACh release from these cells was reduced (Doussau *et al.*, 1998).

Given the findings described above, the characterisation of Rab3GAP in this study produced some unexpected results: myc-tagged p130 Rab3GAP was not found to dissociate coexpressed EGFP-Rab3A from secretory granules, and did not colocalise with granules labeled with EGFP-Rab3A

or with EGFP-Rab27A (Fig.5.1, A-F), or produce any effects on secretion in hGH cotransfection assays (Fig.5.1, G). The absence of any colocalisation between myc-Rab3GAP and granules was not unexpected in itself, because RabGAPs do not always strongly localise to the same membrane compartment as their target Rabs. For example, RN-tre, which is thought to act as a GAP for the Golgi localised Rab43, localises to the cell surface (Haas *et al.*, 2007). However, it was surprising that the protein failed to disperse EGFP-Rab3A or affect secretion, as this suggests that it was unable to promote Rab3A GTP-hydrolysis, at least prior to fusion between secretory granules and plasma membrane.

It is possible that as previously suggested, Rab3A is not susceptible to GAP activity under resting conditions as a result of its interaction with effector proteins (Grosshans *et al.*, 2006). However, it was shown earlier in this study that EGFP-Rab3A is exchanged between granules and cytosol under resting conditions (Handley *et al.*, 2007), a process very likely to require GAP-stimulated GTP hydrolysis. Furthermore, it has been shown that overexpression of RabGAP5 efficiently dissociates EGFP-Rab5 from endosomes, despite the capacity of this protein to form stable domains resistant to GAP activity (Haas *et al.*, 2005; Grosshans *et al.*, 2006). An alternative possibility might be that the availability of its noncatalytic subunit p150 Rab3GAP, and/or the vesicular protein Rabconnectin3, restricts the in vivo activity of p130 RabGAP. In brain tissue, the majority of p130 Rab3GAP is present in a soluble synaptic fraction, while these

factors have been suggested to transiently direct the protein to its site of action (Nagano *et al.*, 1998; Kawabe *et al.*, 2003; Aligianis *et al.*, 2006).

Following the screening of a panel of putative RabGAPs in this study, a novel candidate Rab3-specific RabGAP, RUTBC1, was identified. In PC12 cells, myc-tagged RUTBC1 was found to colocalise with coexpressed EGFP-Rab27A on secretory granules, while it efficiently dissociated coexpressed EGFP-Rab3A from these structures (Fig.5.5, A-F). In subsequent experiments, it was found to suppress the inhibitory effects of EGFP-Rab3A overexpression on secretion of exogenous hGH from these cells, while it had no effect on the inhibition produced by EGFP-Rab27A overexpression (Fig.5.5, G-H). Although RUTBC1 overexpression alone did not affect secretion of cotransfected hGH (Fig.5.5, G-H), GAP-promoted Rab3A extraction from granules is unlikely to be complete, because it does not affect the protein's initial targeting to these organelles, and because the extraction process itself requires GDIα (Sakisaka *et al.*, 2002; Goody *et al.*, 2005). Further, it is likely that in cells overexpressing a Rab3-specific GAP, some fraction of Rab3A would be present on granules in a GAP-resistant state, through interactions with effectors, or through incorporation into hypothesised 'Rab domains' (Grosshans *et al.*, 2006). It is possible, then, that a reduced granule-associated pool of Rab3A is adequate to fulfil its cellular functions. Together, therefore, the findings presented here are consistent with the suggestion that RUTBC1 specifically regulates Rab3 activity. Also consistent with this suggestion,

mRNA expression profiling published on Symatlas (symatlas.gnf.org) suggests that like Rab3, it is strongly expressed in the brain.

Future work will be required to directly characterise RUTBC1 GAP activity, while it is interesting to speculate that RUTBC1 and p130 RabGAP may each regulate Rab3 GTP-hydrolysis, but may act in distinct locations. It has been suggested that RabGAP-5 regulates Rab5 activity associated with early endosome fusion, while RN-tre, although primarily a GAP for Rab41, regulates its function in macropinocytosis (Schnatwinkel *et al.*, 2004; Haas *et al.*, 2005; Pfeffer, 2005a). By analogy therefore, RUTBC1 and p130 Rab3GAP may regulate Rab3 activity on secretory granules and synaptic vesicles respectively, or may differentially regulate Rab3 activity pre- and post-fusion.

5.3.2 Rab27 GTPase-activating proteins

In a recent study, it was suggested that the proteins TBC1D10A and TBC1D10B function as GAPs for Rab27 (Itoh and Fukuda, 2006). These proteins were identified after a panel of putative RabGAPs was screened for their capacity to strongly induce melanosome aggregation, an effect seen in the melanocytes of *ashen* mice in which Rab27A is inactive (Wilson *et al.*, 2000; Itoh and Fukuda, 2006). On subsequent testing, their overexpression was also found to reduce the levels of exogenous GTP-Rab27A that could be extracted from Cos-7 cell lysates, and TBC1D10A was found to show GAP activity towards Rab27A, but not Rab3A, in vitro (Itoh and Fukuda, 2006).

Despite initially appearing conclusive, the data described above, and the experimental procedures used to generate it, may be open to criticism. Where melanosome aggregation was induced by TBC1D10A or TBC1D10B overexpression, melanosomes appeared to adopt a restricted perinuclear localisation (Itoh and Fukuda, 2006). This localisation differs markedly from that in ashen mice, in which melanosomes are more widely distributed within the central regions of cells, and so it may well have had an unrelated cause (Wilson *et al.*, 2000; Wu *et al.*, 2001). Where the effects of the proteins' overexpression on GTP-Rab27A extraction from cell lysates was judged, each of five putative GAP proteins, included in experiments alongside TBC1D10A and TBC1D10B, also appeared to reduce this extraction. Although TBC1D10A and TBC1D10B produced a more dramatic effect, it is notable that their expression levels were greater than those of the other GAPs tested (Itoh and Fukuda, 2006). Where the GAP activity of TBC1D10A towards Rab27A and Rab3A was compared, GTP-hydrolysis was not measured directly. Furthermore, incubation times were varied so that Rab27A was exposed to the protein for 40 minutes, while Rab3A was exposed for only 20 minutes (Itoh and Fukuda, 2006; Itoh *et al.*, 2006). These times were varied to account for the differing intrinsic GTPase activity of the two Rab proteins, though a specific GAP would be expected to accelerate GTP hydrolysis dramatically, and though there is no evidence to suggest that rates of intrinsic and GAP-catalysed Rab-GTP hydrolysis correlate (Clabecq *et al.*, 2000).

When TBC1D10A-myc was expressed in PC12 cells in this study, it was largely localised to the cell periphery, with a similar staining pattern to that previously seen with phalloidin (Fig.5.2, B, E, H, K). This localisation is consistent with reports suggesting that the protein binds to the ezrin-binding protein EBP50, since ezrin is an ezrin/moeisin/radixin family member, and involved in linking F-actin to the plasma membrane (Reczek and Bretscher, 2001; Hanono *et al.*, 2006). When coexpressed with either EGFP-Rab3A or EGFP-Rab27A, the effects of TBC1D10A-myc coexpression were variable. In some cells the exogenous Rab proteins showed characteristic localisation to punctae, while in others, they appeared more diffuse (Fig.5.2, A-L). These data may indicate that TBC1D10A has a relatively promiscuous GAP activity, although the effects of its overexpression do mirror those of siRNA knockdown of Rab27A in terms of reduced secretory responses (Fig.5.2, M)(Waselle *et al.*, 2003; Tsuboi and Fukuda, 2006a).

Like TBC1D10A-myc, overexpressed TBC1D10B-myc was largely localised to the periphery of PC12 cells (Fig.5.3, B, E, H, K). Its overexpression, like that of TBC1D10A-myc, was found to disperse cotransfected EGFP-Rab3A and EGFP-Rab27A with similar, albeit variable, efficiency (Fig.5.3, A-L), and was found to inhibit secretion as judged through hGH cotransfection assays (Fig.5.3, M). There was a propensity of TBC1D10B-myc overexpression to cause the formation of large intracellular vacuole-like structures not seen when TBC1D10A-myc was overexpressed in this study, but this was the only finding presented

here that distinguishes the behaviour of these proteins (Fig.5.3, E, K). In recent work elsewhere, TBC1D10B was screened for GAP activity against a panel of 46 different Rab proteins, and was found to have in vitro activity against Rab22a, Rab31 (22b), and Rab35, while it showed no activity against Rab27A (Fuchs *et al.*, 2007). This report not only draws into question the previous characterisation of TBC1D10B as a Rab27GAP, but because of their similarities, may also draw into question that of TBC1D10A.

In screening experiments carried out in this study, several additional candidate GAPs for Rab27A were identified. RUTBC2-myc showed some capacity to disperse EGFP-Rab27A but not EGFP-Rab3A when coexpressed with these proteins. However, it did not colocalise well with EGFP-Rab3A, and its overexpression did not affect secretion, or suppress the inhibitory effects of EGFP-Rab27A overexpression on the process (Fig.5.6). These data may not give a strong indication that the protein possesses Rab27GAP activity, but equally, they do not rule out this possibility. Future work may be directed towards clarification of this point, along with characterisation of the other potential Rab27GAPs, TBC1D3 and AK_074305.

Chapter 6:

Summary and discussion

6.1 Summary and discussion

The work described in this study characterised several aspects of Rab3A recruitment and dynamics in PC12 cells, and may indirectly support a speculative model for its regulation. It was shown through fluorescence microscopy, immunocytochemistry, and 'temperature-block' experiments that Rab3A was preferentially recruited to newly synthesised secretory granules, a process beginning between 10 and 20 minutes after exit of immature granules from the TGN. FRAP experiments showed that at least a component of cellular Rab3A cycles between secretory granule membranes and cytosol under resting conditions. Further, the rate of this cycling was found to increase slightly over time, and to occur in an HSP90-independent manner. In a series of hGH cotransfection assays, EGFP-Rab3A expression, but not HSP90 inhibition, was found to inhibit secretion, and in live-cell imaging experiments, exocytosis of secretory granules was not found to be associated with the dispersal of the protein. These data may support a model in which the recruitment of Rab3A can lead to its stable association with granules through interaction with GEF protein in a multimolecular 'Rab domain', as is the case for Rab5A where it associates with endosomes (Zerial and McBride, 2001; Grosshans *et al.*, 2006). Sequestration of Rab3GEF in domains containing Rab3A may be accompanied by a reduction in the capacity of granules to bind additional cycling Rab3A. Also, the disassembly of these domains following exocytosis and Rab GTP-hydrolysis may require an additional factor(s).

Like Rab3A, Rab27A was found to be preferentially recruited to newly synthesised secretory granules, its overexpression was found to inhibit secretion, and it was not found to disperse on cell stimulation. However, FRAP experiments showed that unlike Rab3A, it formed a stable association with secretory granules once recruited. Additionally, an ECFP-Rab27A Q78L 'constitutively active' mutant protein was found to be largely cytosolic, though a fraction of the protein did associate with secretory granules, and its overexpression did inhibit secretion. Speculatively, these data may support the suggestion that Rab27A may saturate stable binding sites on new granule membranes, and subsequently be protected from GDI-mediated membrane extraction following GTP hydrolysis. A possible mechanism for such protection might be incorporation of the Rab into a highly stable Rab domain, or dimerisation of the GDP-bound Rab as has been suggested for Rabs 9A, 11A and 27B (Pasqualato *et al.*, 2004; Wittmann and Rudolph, 2004; Chavas *et al.*, 2007). The Q78L mutation may reduce Rab27A membrane association by reducing its affinity for its GEF, or its capacity to dimerise.

When the localisation, dynamics and function of Rab effectors was explored, Noc2 overexpression was found to produce the most pronounced effect on secretion in PC12 cells, and through FRAP experiments, the protein was found likely to interact with secretory granules in a sustained manner. Mutational analysis supported previous suggestions that Noc2 is recruited to secretory granules via Rab27A in PC12 cells (Haynes *et al.*, 2001; Cheviet *et al.*, 2004a; Fukuda *et al.*,

2004). Rabphilin and Granuphilin also localised to secretory granules, although Granuphilin preferentially localised to granules at the cell periphery. Because Granuphilin was largely granule associated, but showed some degree of exchange between granules and cytosol, it is possible that this localisation was the result of binding to both Rab27A, and an additional factor at the plasma membrane, possibly Munc18, Syntaxin or PIP_2 (Coppola *et al.*, 2002; Torii *et al.*, 2002; Torii *et al.*, 2004; Fukuda *et al.*, 2005; Gomi *et al.*, 2005; Tsuboi and Fukuda, 2006b; Osborne *et al.*, 2007). It is also possible that such interactions promoted the redistribution of granules to the cell periphery as previously described (Gomi *et al.*, 2005). The exchange of Rabphilin between granules and cytosol was very rapid, possibly the result of repeated rounds of Rab3A or Rab27A GTP-hydrolysis and reassociation at the plasma membrane. Coupled a previous study apparently showing that Rabphilin improves granule mobility at the plasma membrane, and studies showing that it can interact with the actin bundling protein α-actinin, this finding may be consistent with a model in which Rabphilin locally remodels subplasmalemmal cortical actin to promote the passage of granules through it (Kato *et al.*, 1996; Baldini *et al.*, 2005; Tsuboi and Fukuda, 2005).

When an E466K mutation was introduced into the Syntaxin-binding protein Munc18-1, it allowed the discovery that this protein also interacts with Rab3A (Graham *et al.*, 2008). Expression of Munc18-1 bearing the E466K mutation was shown in this study to promote peripheral localisation of

newly synthesised secretory granules labelled with either EGFP-Rab3A or EGFP-Rab27A. The effects of the mutant protein were consistent with recent work in this laboratory showing that its overexpression enhanced adrenal chromaffin cell secretory responses (Graham *et al.*, 2008). Given findings showing that Munc18-1(E466K) interacts more strongly with Rab3A than the wild-type protein, these data also suggest that the interaction between these proteins promotes calcium-regulated exocytosis. On the basis that a Munc18-1(E466K/R39C) double mutant, capable of interacting with Rab3A in vitro, but binding with reduced affinity to the 'closed' conformation of Syntaxin, was unable to promote peripheral granule localisation, or secretion, it may be speculated that a Rab3A-Munc18-1-Syntaxin interaction facilitates controlled assembly of trans-SNARE complexes. Testing of this hypothesis may be important in future work, if transient interactions between Rab and SM proteins represent a conserved mechanism for Rab function in membrane fusion across multiple trafficking pathways, as is consistent with some of the data available from studies in yeast (Dascher *et al.*, 1991; Ossig *et al.*, 1991; Ballew *et al.*, 2005).

The regulation of Rab3A and Rab27A GTP-binding was investigated in this study through analysis of proteins reported to show GAP activity towards them, and of a panel of other putative RabGAPs. It was found that the previously studied p130 Rab3GAP (Fukui *et al.*, 1997) was not able to disperse granular EGFP-Rab3A or EGFP-Rab27A on its overexpression, and that it adopted a different localisation in PC12 cells, perhaps indicating

that it is not the major physiological Rab3GAP. TBC1D10A and TBC1D10B, which have been reported to act as GAPs for Rab27A (Itoh and Fukuda, 2006) were each found to indiscriminately disperse both EGFP-Rab3A and EGFP-Rab27A in a proportion of PC12 cells, while they localised to the cell periphery, possibly to the cortical cytoskeleton. This study identified RUTBC1 as a potential Rab3GAP on the basis of its dispersal of EGFP-Rab3A in over 75% of coexpressing cells, its colocalisation with EGFP-Rab27A on secretory granules, and its capacity to suppress the inhibitory effects of EGFP-Rab3A on secretion. Further work including direct analysis of its effect on Rab3A GTPase activity will be required to substantiate the possibility that RUTBC1 is the bona fide Rab3A GAP. In addition, the identity of the Rab27A GAP still remains to be determined.

To conclude, it has been shown here, that Rab3A and Rab27A interact with secretory granules in a dynamically distinct manner, and that the ways in which their diverse effectors are recruited and released may be similarly distinct. It has been suggested that Rab and SM protein interactions may coordinate membrane fusion events, and a putative Rab3GAP has been identified and partially characterised. This study supports a view in which Rab proteins dynamically establish asymmetry on the communication between membrane compartments, and in which this asymmetry is conferred in the form of arrayed, ephemeral, multimolecular assemblies, and the delicate transitions between them. It is hoped that their characterisation will continue.

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